Canada. Agriculture, Department of. Bacteriology Division
Qualitative studies of soil microorganisms I-XV (1938-1957)

QUALITATIVE STUDIES
OF
SOIL MICROORGANISMS

I-XV (1938-1957)

BACTERIOLOGY DIVISION
SCIENCE SERVICE
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OTTAWA

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Qualitative Studies of Soil Microorganisms

FOREWORD

During the years 1938 to 1957 a series of investigations carried out by members of the Bacteriology Division was published in fifteen papers under the general title "Qualitative Studies of Soil Microorganisms." As described more fully in the introductory paper, the purpose of the work was to study the indigenous bacteria as such rather than as agents of known processes. This type of investigation, directed to a better understanding of the nature of soil microorganisms, their reactions to environmental influences and, eventually, their role in soils, has received scant attention compared to that given to studies of processes in which organisms are known to participate. The work attempted, therefore, to strike a better balance between what might be termed the ecological and the functional approach to soil microbiology.

In view of the continued interest of many research workers in this series of publications, most of which are no longer available in reprint form, it is hoped that having this group of studies under one cover will be helpful to many engaged in research in soil microbiology and related fields.

QUALITATIVE STUDIES OF SOIL MICRO-ORGANISMS

I. GENERAL INTRODUCTION[1]

By A. G. Lochhead[2] and C. B. Taylor[3]

Abstract

Soil microbiological research has been directed for the most part towards a study of processes in which micro-organisms are known to participate rather than towards an objective study of soil micro-organisms themselves. While organisms concerned with known processes have been given much study, relatively little attention has been paid to groups of bacteria whose functions are as yet unknown or but little understood, but which are believed to comprise a very large proportion of the micro-population of arable soils. A review is made of investigations based on the biological, as contrasted with the biochemical (or functional) approach to soil microbiology. Qualitative studies of the general soil microflora are regarded as essential to a better understanding of microbiological activity in soil and its relation to practical problems of crop growth, soil borne plant diseases, and general soil fertility.

Approach to Soil Microbiology

Soil microbiological research in the main has been directed towards studying microbiological processes rather than the micro-organisms themselves. The rise of bacteriology in the latter half of the nineteenth century led to an immediate and phenomenal application to medicine which gave hopes of an equally effective application of the new science to problems of soil fertility. Coincident with the discoveries, in the closing decades of the century, of the role of bacteria in human and animal disease, equally brilliant if less spectacular discoveries were made of the part played by micro-organisms in many soil processes.

Right up to the present the study of processes, and incidentally that of the specialized groups of organisms concerned in these processes, has occupied by far the greater part of the attention of most soil microbiologists. Valuable data have been gathered on the numerous biological processes known to occur in soils, such as ammonification, nitrification, nitrogen-fixation, processes concerned with the transformation of sulphur and other elements, the decomposition of plant residues and miscellaneous organic compounds. Detailed investigations have been made of bacteria and other micro-organisms known to take part in such processes. However, such organisms have been studied, not so much from an interest in them as organisms, but because of their known, and presumably important functions.

The immediate application of bacteriology to medicine, and a similar concentration, in the case of soil microbiology, on functions, has if anything delayed progress in the objective study of bacteria. Even today, when microbiology is so widespread in its application, many of the fundamental questions of the morphology and physiology of bacteria remain unanswered or at least in active dispute.

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Contribution No. 47 (Journal Series) from the Division of Bacteriology, Dominion Experimental Farms, Ottawa.
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In soil, bacteria are indigenous to the medium in a sense not true of bacteria in milk, in foodstuffs or in blood or living tissues. In arable soil we have a centuries-old equilibrium, though admittedly an ever-changing one, not analogous to conditions prevailing, *e.g.*, in a given sample of milk or in an infected animal. In the latter instances, too, we can better recognize cause and effect; we have to deal with fewer antagonisms and associations of different groups of organisms; and we have a better conception of the functions of the organisms in the medium. In the soil we can perceive many biological processes. Some of these we can evaluate fairly well; others, such as non-symbiotic nitrogen-fixation, we are unable to evaluate though the organisms able to exercise the function under artificial conditions have been given much study. There is reason to believe, however, that the organisms in soil which take part in recognized soil functions are greatly outnumbered by those whose functions are yet unknown.

Greater cultural difficulties doubtless stand in the way of a biological, as contrasted with a biochemical (or functional) approach to soil microbiology. We require a non-selective rather than a selective medium to permit of the isolation and study of the greatest numbers of types occurring in soil. Much more success has been achieved in the development of highly selective culture media than in the development of non-selective media, and of the latter type the most we can say is that they are less selective than the others.

Our least selective media are used for the most part for "total counts" of soil organisms. The enumeration of plate colonies constitutes the only consideration usually given to many types of micro-organisms characteristic of soils and forming a large part of their total population. The importance of many types in soil is likely to be gauged by the size of their respective colonies, very many of which are small or of pin-point size. This impression may be strengthened by casual microscopic and physiological tests which indicate, in a large number of cases, small, rod-shaped organisms, relatively inactive as judged by our standard artificial testing procedures. At the present state of our knowledge, however, micro-organisms that do not appear to fit into our more important physiological groups as we recognize them today, cannot be dismissed as unimportant. More study of an objective nature is required before we can reliably assess such groups. Their undoubted abundance in most soils renders essential a more thorough knowledge of them than we possess at present; such knowledge would help to form the basis of a more complete understanding of soil micro-organisms in general.

The General Bacterial Microflora of Soil*

Our present knowledge of the general microflora of the soil, as distinct from types of micro-organisms concerned with known biochemical processes, is due to a comparatively small number of investigators, and in a large measure

* *This paper is concerned primarily with bacteria, the most abundant soil organisms and the group studied relatively least from the purely qualitative point of view. It is recognized that actinomyces, fungi, algae and protozoa comprise important divisions of the micro-population of soil.*

to the work of Conn, who extended and developed ideas underlying the earlier work of Chester. Previous investigators, to be sure, had reported studies of bacteria isolated from soil. As early as 1881, R. Koch (26) made the observation, which has been repeatedly confirmed since, that rod forms are greatly in excess of cocci. As was but natural from the prominence of their colonies on beef gelatine or agar plates largely employed in earlier work, spore-formers received particular attention. Houston (21) describes various types of this group, devoting less attention to non-spore-forming organisms. He and other contemporaries of Chester, such as Gottheil (19) and Neide (31) who studied spore-formers, were apparently more interested in definite groups than in the whole microflora of soils.

In 1900 Chester (1) first emphasized the importance of a knowledge of the types of organisms predominating in soil. Introducing a study on bacterial classification he enunciated a principle which has received all too scant attention.

"Agricultural bacteriology is destined to have a very important bearing, but as yet is without any foundation. The animal pathologist deals with a comparatively few forms which he can readily identify. The agricultural bacteriologist, on the other hand, can scarcely take up a piece of work before he meets with scores of bacterial forms of which he knows nothing, and which he is unable to identify. Hence the first desideratum before he can advance in this important field is to possess some system of bacterial classification, however crude and imperfect. These studies in bacterial classification have been preliminary to the investigation of the bacterial flora of cultivated soils. Inasmuch as soil bacteria are the active agents for the digestion and elaboration of plant food in soil it is important to know something about them, not only collectively but individually. It is necessary to know what species of bacteria are commonly present in all soils and the part each plays in plant food elaboration."

In the same year Chester (2) described miscellaneous types of bacteria isolated from soil, and in 1903 (3) published what is probably the first study of the predominating bacteria of soil. From gelatine plates of 1/100,000 dilution of soil, giving but small numbers of colonies, the predominating types of organisms were studied in detail. The three main types in order of abundance were named, following Migula's system of classification, *Streptothrix soli*, *Bacterium flocosum* (a non-motile spore-former) and *Bacillus Delavariensis* (a motile non-spore-former). This and related studies by Chester (4), such as a special investigation of spore-forming bacteria in soil, represent the most important work up to that time on the qualitative nature of the soil microflora. It was pointed out by the same author (5) that in order to form a true estimate of what is taking place in soil through the agency of bacteria we should understand the function of the different types. He stressed the importance of isolating all types which predominate in soil and of a quanti-qualitative soil microbiological analysis.

Chester was breaking new ground while bacteriological methods were still far from perfect. The principles involved in his work are perhaps more

important than the findings obtained, and deserve more consideration than has been accorded up to the present.

In 1903 Hiltner and Störmer (20) made a study of types of bacteria in soil, on lines of general groups rather than of definite species. Three main groups were recognized, liquefiers, non-liquefiers and Streptothrix. The numerical importance of spore-formers in soil was put in doubt by studies which showed that they comprised but a small percentage of colonies on plates whereas non-liquefying, non-spore-forming organisms formed by far the largest group. Whereas Chester (5) inclined to the belief that the prevalence of kinds of bacteria in a soil was largely a fortuitous matter, with relatively few species predominating, Hiltner and Störmer found the relative numbers of the broad groups to be fairly constant in normal soils, suggestive of a certain state of equilibrium.

In a series of studies first appearing in 1917, Conn (7, 8, 9, 10, 12) added much to our qualitative knowledge of soil bacteria through extensive work on the general soil flora as contrasted with the more intensive work on special groups of organisms that were considered important on account of their physiological activities and commanded most attention from contemporary soil biologists. Conn's provisional classification recognized five main groups in soil as determined by studies of colonies on gelatine and agar plates:— (i) spore-formers, (ii) rapidly liquefying, non-spore-forming short rods, (iii) slowly liquefying or non-liquefying, non-spore-forming short rods, (iv) micrococci and (v) Actinomyces. Of these, Groups (ii), (iii) and (v) were the most numerous, the slowly liquefying or non-liquefying short rods being the most abundant and doubtless comprising the same broad group recognized by Hiltner and Störmer. Martin (30) likewise found non-spore-formers to comprise the majority of organisms in normal soil with Actinomyces next in abundance, and spore-formers occurring in smaller numbers. In a study of Texas soils, however, Williams (38) reported having found spore-forming bacteria as the dominant types from an examination of colonies isolated. However, as no attempt was made to determine the relative abundance of different forms occurring on plates it is not possible to assess the relative incidence of the various types.

In studying the predominant types occurring in frozen soil Lochhead (29) showed the largest group to consist of slowly liquefying or non-liquefying, non-chromogenic short rods, which group represented in even more pronounced degree the majority of bacteria capable of growth at low temperature (3° C.). Actinomyces, though unable to grow at low temperature, comprised the second largest group. Non-spore-forming, liquefying short rods, spore-formers and micrococci formed numerically much less important groups. The relative abundance of the different groups in frozen soil corresponded closely with the findings of Conn and led to the belief that, as far as the main types are concerned, the winter flora of soils differs little from the summer flora.

The rapidly liquefying short rods, Group (ii) of Conn's classification, were apparently closely related to *Pseudomonas fluorescens*. Though forming a

relatively small portion of the total microflora, they were found by Conn to be specially abundant in freshly manured soil, a finding confirmed by the work of Lewis (28), and with their distinctly proteolytic properties were suggested as being important soil ammonifiers.

The great majority of the non-spore-forming organisms, made up largely of the group of slowly liquefying or non-liquefying short rods, were less active physiologically and grew less abundantly on the media used. Organisms of this group were referred to at first as "slow growers" by Conn, and later as "punctiform colony forming bacteria" on account of the restricted size of colonies on tap water gelatine. While several sub-divisions of this group were made (14), special attention was given to two types which embraced the great majority of the forms studied:

(I) Small, short rods, motile or non-motile, with no tendency to produce irregular forms. With this sub-group many variations in staining properties and physiology may be observed. This suggests either the existence of many species within the sub-group or unstable physiological characters.

(II) Pleomorphic forms, appearing as short rods in young culture but changing into cocci within a few days. While variability in staining and physiology occurs, it is less pronounced than in the previous sub-group.

In addition to the above, other much less abundant sub-groups (III and IV) were noted, less definitely classifiable, but showing a tendency to produce filaments, branched or unbranched. These forms are doubtless related to soil organisms showing branching and described as members of the genera *Corynebacterium*, *Mycobacterium* and *Proactinomyces* by Jensen (22). It is probable that such organisms with tendency to produce branched forms may comprise relatively large proportions of the micro-population in some soils. Thus in Australian soils Jensen (23) found corynebacteria to comprise from 8 to 65% of the colonies appearing on dextrose agar plates. *Mycobacteria*, however, were found by Jensen (24) to occur much less frequently, though Krassilnikow (27) states that they are widely distributed in certain Russian soils, especially those rich in humic substances.

Organisms of the sub-group (II) above, comprising the cocci-forming rods, gave evidence of a much closer inter-relationship than those of (I) and were regarded as consisting almost wholly of one species, to which the name of *Bacterium globiforme* was given. To this organism, one of the predominating types in the soils studied, special attention has been given by Conn (13, 14) and Conn and Darrow (16, 17), particularly in view of its apparently greater abundance in many productive soils than in certain less productive soils investigated.

Even in soils that may be classed as abnormal the main groups recognized by Conn appear to be present, though under extreme conditions such as are represented by arid or desert soils the proportions may be considerably altered. In a series of investigations summarized by Snow (33) studies were made of the bacterial flora of wind-blown soils from six localities. In but one of the soils studied was the average total count in excess of 100,000 per gram.

Examination of the types isolated suggested that cocci, liquefying short rods, and in most cases spore-forming rods, formed relatively larger proportions of the cultures from such soils than of those from more arable soils.

Coincident with progress in soil flora studies from the cultural side has been a development of methods for the direct microscopic study of soil organisms. The first method, proposed by Conn (11), was essentially an adaptation of the Breed smear method for milk examination, and consisted in the staining of a suspension of soil after fixing and drying on a slide. This procedure, with some modification, was later used by Winogradsky (40, 41) in connection with his "direct method" of soil study. A very important development was made by Rossi and Riccardo (32) who first advocated the use of the direct contact slide method and provided a new means of studying not only the forms, but also colonies of micro-organisms, as they occur in soil, and other points of interest not brought out by the stained suspension method. A very important modification of this, the most direct method, was made by Cholodny (6), while further adaptations were suggested by Conn (15) and others for the examination of soil *in situ* or in the laboratory, with any desired modification or treatment.

By the aid of the microscopic method, Winogradsky (39, 41) was able to study the main morphologic types in soil and particularly their reaction to changes in environment, such as are caused by the addition of nutrient materials. He classified soil organisms in two main categories. One consists of the *autochthonous* (*i.e.*, indigenous) flora, characteristic of soils poorly supplied with fermentable substances. Organisms of this group are for the most part oval forms or cocci, comparatively inactive and believed to take part in the slow combustion of the humic constituents of soil. Another category was recognized which he calls the *zymogenic* organisms. These are scarce in normal soils but become very active upon addition of any readily fermentable substance. In this group are included the spore-formers, which as Joffe and Conn (25) had shown, are apparently inactive under ordinary field conditions but may multiply upon addition of fresh available organic matter, particularly when abundant moisture is present, and engage in decomposition processes.

Apparently Conn's group of non-spore-forming bacteria corresponds essentially to Winogradsky's autochthonous group, representing the indigenous soil organisms as contrasted with other types which come into prominence mainly under special conditions. The agreement at first was not so evident. Winogradsky assumed that the autochthonous group was largely incapable of being cultivated on ordinary media from the fact that, whereas cocci forms predominated in the microscopic examination of soil, relatively few cocci developed in cultures. However, Thatcher and Conn (35) found that in some soils as many as 40% of the organisms growing on plates may consist of coccus-forming rods. This work was followed by the studies of Conn, and Conn and Darrow, referred to above, and by the recognition of the *Bacterium globiforme* group as an important part of the autochthonous soil flora.

Further application of the microscopic method has been made by various workers in studying the relative abundance of different groups in soil, particularly as affected by soil treatment. Thus the work of Demeter and Mossel (18) and of Vandecaveye and Villanueva (37), though carried out by different modifications, showed that useful application could be made in indicating qualitative changes on a broad basis with approximative quantitative values. The method, however, is inadequate for studies of the role of the organisms in soil. The soil slide method provides us, to be sure, with an additional and valuable means for soil flora investigation. It possesses certain advantages and also the limitations of microscopic methods. From a qualitative point of view it may be used to advantage in noting the prevalence of different morphologic types and their reaction to environmental changes. It is therefore a useful supplement to cultural studies, but the latter, however, are necessary for an adequate study of the unknown organisms in soil and their possible functions.

Of the autochthonous microflora it would appear that *Bacterium globiforme* (or the *Bact. globiforme* group) comprises a significant part, though relatively little attention has been accorded it. Conn (13, 14, 16, 17) studied the physiological properties of the organism and furthermore noted certain relationships between its incidence and soil productivity. Of interest was its occurrence in certain fertile soils and its absence from certain less productive soils, suggesting that the inability of the latter to support growth of *Bact. globiforme* was associated with their relatively low crop-producing ability. The work of Conn and Darrow (16) suggested further that the growth of the organism in soil was dependent upon the presence of readily available nitrogen, which is lacking in the poor soils. Further work by the same authors (17) led to the conclusion that the organism retains, in the soil, nitrogen that has been converted by other organisms into a soluble form and which otherwise would be removed by drainage or utilized by plants. Depending upon conditions, therefore, the organism may be beneficial or harmful, with the beneficial function predominating.

In comparing the incidence of *Bact. globiforme* in soils differing in fertility, Taylor and Lochhead (34) found, by quanti-qualitative methods, no indication of relationship between the abundance of the organism and the productivity of the soils in question. There was more indication of an influence of the crop on the bacterium, though in all cases it formed a significant part of the total microflora. The findings obtained, when compared with those of Conn and Darrow, suggest that inability of certain soils to support growth of *Bact. globiforme* may be related to some special factor affecting productivity and not to general lack of crop-producing power.

The most recent study of the predominant micro-organisms in soils is that of Topping (36), who made an examination of the organisms which occurred most numerously in a series of soils from southeastern Scotland and Saxony. Gram-positive, non-spore-forming, non-acid-fast rod forms were found to outnumber all other types, as determined by a study of organisms growing

from the highest dilutions of soil on a variety of media. These Gram-positive bacteria were divided into three groups: (1) motile organisms producing branching variants, (2a) non-motile non-branching rods and (2b) non-motile, mycelium-forming types. All three groups represented forms exhibiting considerable pleomorphism. Organisms of Groups 1 and 2a particularly showed that the production of coccoid from rod forms closely resembled the morphological change undergone by *Bact. globiforme*, and the author considers strains of Group 2a to be probably related to this organism. This group is moreover believed to be related to *Corynebacterium*, while from their form members of Group 2b are considered to belong to Jensen's genus *Proactinomyces*. Though members of the motile Group 1 were not identified with known species the similarity in morphological and cultural behavior shown by strains of Groups 1 and 2 suggests a close relationship to the *Proactinomycetaceae*, motile species being recognized in both *Corynebacterium* and *Proactinomyces*. Gram-negative rods, classed in Group 3, were found to be less conspicuous than the Gram-positive forms. They were in the main chromogenic forms, and although they did not undergo the striking morphological changes exhibited by the Gram-positive types, they resembled the latter in their general biochemical inactivity.

In considering the evidence from qualitative studies so far reported a number of points seem to be established:

(1) In arable soils a large proportion of the bacterial flora consists of organisms whose functions are unknown or but little understood.

(2) Under normal soil conditions the predominant types of organisms are non-spore-forming short rods, motile or non-motile, cocci and spore-forming bacteria being relatively insignificant.

(3) Many predominant soil species are highly pleomorphic. Included in these are the *Bacterium globiforme* group and organisms closely related to corynebacteria and mycobacteria.

(4) The majority of soil bacteria are relatively inactive physiologically as judged by standard laboratory tests. This by no means excludes the possibility of important biological activity in soil.

The work so far done points to the value of more extensive studies of the qualitative nature of the soil microflora and the types predominating. Only when the autochthonous organisms are known more thoroughly will it be possible to learn their true functions in soil. Such knowledge is regarded as essential to an understanding of general microbiological activity in soil. In view of the intricate systems of symbiosis and antibiosis, it should help in evaluation of the known processes and form a basis for better appreciation of the relation of micro-organisms to growing plants, to soil borne plant diseases, and to soil fertility in general.

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QUALITATIVE STUDIES OF SOIL MICRO-ORGANISMS

II. A SURVEY OF THE BACTERIAL FLORA OF SOILS DIFFERING IN FERTILITY[1]

By C. B. Taylor[2] and A. G. Lochhead[3]

Abstract

Investigations were made, on a non-selective basis, of the qualitative nature and relative incidence of the different types of the bacterial flora of three soils differing in fertility. The organisms were classified into eight groups. Non-spore-forming short rods, of which five groups were recognized, comprised nearly 90% of all types. Gram-negative short rods formed the most prevalent single group, rather more numerous than Gram-positive short rods. Gram-variable short rods, coccoid rods and pleomorphic rods *(Bact. globiforme)* were regarded as definite groups. Cocci, non-spore-forming long rods and spore-formers were less prominent soil types.

In spite of unequal productivity, the soils showed no outstanding differences in the relative incidence of the bacterial groups. Certain groups showed some indication of seasonal and cropping effect. The results suggest that the general character of the *autochthonous* (indigenous) soil flora is relatively uniform in soil of definite type, even though productivity may be greatly altered by manurial treatment.

The predominant soil bacteria appear relatively inactive in single culture. Moreover considerable divergence in biochemical action was shown by apparently closely related forms. It is suggested that the bacterial flora is relatively unstable physiologically, with considerable adaptability, and that the functions of the different species are exercised most fully only under conditions of association.

Introduction

The present paper is one of a number of studies on the qualitative nature of the microflora of soils, most of the relevant literature of which has been discussed in the first paper of this series (6). The object of the investigation was to study, on a non-selective basis, the bacterial types occurring in three soils, and their relative incidence. The soils were of similar type and crop history, but by reason of different fertilizer treatment for 25 years they had become widely dissimilar in productivity. A previous study (7) had been made of the abundance of certain strains of *Rhizobium* and *Azotobacter* in these soils, while as a side issue in the present work the incidence of *Bacterium globiforme* has been reported earlier (10).

Experimental

The soils studied were taken from three plots of different manurial treatment in a four-year rotation system of oats, clover, timothy and mangels. For the preceding 25 years the plots had been receiving the following treatments:

Soil N—no fertilizer

Soil X—15 tons farmyard manure, applied to mangels

Soil Y—100 lb. nitrate of soda, 300 lb. superphosphate, 75 lb. muriate of potash to mangels; 100 lb. nitrate of soda to oats, clover and timothy.

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The soils were of a sandy-loam nature and contained approximately 0.11%, 0.16% and 0.13% nitrogen respectively. The pH value fell between 7 and 8 with variations depending on crop, treatment and season. As may be noted from Table I, striking differences in fertility exist between treated and untreated plots. Mangel yields show this most clearly. The soils thus included an originally fertile soil impoverished by continuous cropping (N) and soils maintained at good fertility levels by farmyard and inorganic manures respectively (X and Y).

Composite samples were taken from the 2–4 in. layer, September 22, 1936, for preliminary tests, and on November 12, 1936, February 23, 1937, April 16, 1937, and July 21, 1937, from plots which had produced a crop of timothy and which at the time of the July sampling were supporting a mangel crop. In September and November, samples were likewise taken from corresponding plots after a crop of mangels. The February sampling represented frozen soil; but at the time of the April sampling the soil had thawed to a depth of 6 inches. Samples were analyzed as soon as possible. Plate cultures of 1/500,000 dilution were prepared. For comparison total cell counts were made by the ratio method of Thornton and Gray (12).

Cultural studies of the general microflora require a medium as non-selective as possible. For this reason, soil extract agar prepared according to Löhnis (8), and without added energy material, was chosen in preference to other media which, though synthetic and hence more easily reproducible, are regarded as more selective on account of the special energy sources contained. The advantage of soil extract was noted in previous tests (9), which gave higher counts with it than with more selective media such as Thornton's (11) mannitol-salts medium. From each sample five replicate plates were poured and incubated at 28° C. for 12 days before counting.

It is felt that the value of any qualitative study is enhanced when quantitative aspects are also taken into account. This is done not only by using definite dilutions, but by applying quantitative methods to an examination of the colonies. Haphazard selection or assumption of similar identity from microscopic observation are unsuited to an estimation of the relative incidence of different types. All colonies on a plate or on a definite sector should be examined on plates with a reasonable number of colonies. When few colonies are present the chances of error by regarding a possible contaminant as predominant are greatly increased. From representative plates all colonies on a sector containing approximately 60 bacterial colonies were picked and stab cultures made into soil extract semi-solid (containing 0.02% K_2HPO_4, 0.01% yeast extract and 0.3% agar). Preliminary tests indicated that many isolated strains, particularly from small colonies, refused to grow on various other media tested. The use of soil extract semi-solid not only assured the survival of 92% of the strains isolated but permitted a certain differentiation of type.

For group classification the cultures were examined for morphology and reaction to the Gram stain. To detect pleomorphic types of the *Bacterium*

globiforme group which appear first as rods and later change to cocci, observations were made on fresh transfers and on the same cultures at later stages of incubation. Standard agar was found to be an aid in the recognition of this group, though it was unable to support growth of many forms isolated. Physiological tests included gelatin liquefaction, nitrate reduction and dextrose utilization. For the nitrate reduction test semi-solid soil extract with 0.1% KNO_3 was used and for dextrose utilization soil extract semi-solid with 1% dextrose and indicator. The latter medium, being comparatively weakly buffered, was found to be more sensitive to changes than the usual media with peptone.

Plate and Total Cell Counts

A comparison of the three soils from the standpoint of "total numbers" is made in Table I in the form of summaries of the plate and cell counts. The usual seasonal fluctuation observed by so many previous workers is noted. The untreated soil appears less subject to fluctuation in numbers than the treated areas, particularly in cell counts. It is of interest to note that, although the plate count indicated that numbers were well maintained in the frozen (February) soil, cell counts showed in all three cases a drop from the November sampling. The results fail to show any relation between numbers and crop yield. In the case of the soils sampled after mangels particularly, there was little variation in numbers of organisms between the unfertilized and the fertilized areas, in spite of very large differences in crop-producing ability.

TABLE I

TOTAL CELL AND PLATE COUNTS

(millions per gram dry soil)

	Following timothy			Following mangels		
	N	X	Y	N	X	Y
Yield (tons per acre)						
av. 25 years	2.01	3.10	2.66	7.99	22.72	20.91
Yield in 1936	1.65	2.95	2.51	2.59	29.03	25.12
Total cell count						
September	741.2	990.1	2065.9	2403.6	2380.7	2332.6
November	1144.5	1846.8	2524.9	2241.4	2534.5	2166.0
February	982.3	1524.9	1018.5	—	—	—
April	681.3	1071.5	925.3	—	—	—
Plate counts						
September	57.0	52.2	50.8	76.6	109.2	107.9
November	92.6	95.4	116.8	123.1	139.1	126.4
February	90.0	111.3	132.7	—	—	—
April	72.4	81.1	60.0	—	—	—
July	36.3	60.1	70.6	—	—	—

Main Morphological Groups

From the morphological and Gram-staining reactions of the cultures in semi-solid soil extract eight main subdivisions of the isolated organisms were made:

Group I Short rods, Gram-positive
Group II Short rods, Gram-negative
Group III Short rods, Gram-variable
Group IV Short rods, changing to cocci (*Bact. globiforme* group)
Group V Coccoid rods, Gram-positive
Group VI Cocci, Gram-positive or negative
Group VII Long rods, non-spore-forming
Group VIII Spore-forming rods

In some instances difficulty was found in allocating organisms to groups, particularly in the case of certain Gram-positive short rods where the differentiation between rod and coccus was nearly impossible. Such types have been grouped as coccoid rods. Subsequent physiological tests provided some justification for the separate grouping of these forms. Short rods that later became cocci and conformed generally to *Bact. globiforme* Conn have been classified under this head. Short rods that showed no tendency to form cocci were divided into three groups according to their reaction to the Gram stain. The percentage distribution of the various groups in the three soils following timothy and sampled at four seasons is shown in Table II. The cultural characteristics of the various groups are summarized separately for the three soils and given in Tables III, IV and V.

Short Rods

In line with findings reported by a number of previous workers (1, 5, 13) non-spore-forming short rods were found to comprise a large proportion of organisms capable of being isolated from soils. The five groups into which short rods were classified made up 86.7%, 89.6%, and 89.1% respectively of cultures isolated from Soils N, X and Y, a surprisingly close agreement in soils differing so widely in productivity.

Gram-negative short rods. Gram-negative short rods were found to be the most prevalent single group of organisms, being rather more numerous than Gram-positive short rods in each soil, taken over the course of the four sampling periods. The difference was less pronounced in Soil N than in the fertilized soils X and Y. Variation in relative numbers was noted at different seasons. Topping (13) reported the Gram-negative group to be much less frequent than Gram-positive types in soils studied by her. As is seen in Tables III to V this group is the least active physiologically and is suppressed to the greatest extent by modifying soil extract through the addition of dextrose, 42 of 226 strains being entirely inhibited by 1% concentration. It is possible that differences in proportion of groups found may be due to employment of different media for isolation. Some of those employed by Topping may be

TABLE II

MAIN MORPHOLOGICAL GROUPS AT DIFFERENT SEASONS

(Soils following timothy)

	November			February			April			July		
	N	X	Y	N	X	Y	N	X	Y	N	X	Y
Soil moisture, %	15.7	17.2	18.1	24.1	32.3	26.2	24.9	23.1	26.1	13.5	15.9	17.6
Total cultures isolated	59	61	61	62	62	63	64	64	64	41	43	41
Percentage showing no growth on transfer	8.5	13.1	13.1	6.5	3.2	6.4	6.3	3.1	17.2	0.0	11.6	4.9
Percentage showing growth on transfer	91.5	86.9	86.9	93.5	96.8	93.6	93.7	96.9	82.8	100.0	88.4	95.1
Morphological groups	%	%	%	%	%	%	%	%	%	%	%	%
Short rods, Gram-positive	24.0	28.3	35.8	39.6	46.6	30.5	28.3	17.7	9.4	24.4	28.9	20.5
Short rods, Gram-negative	37.0	26.4	32.0	25.8	15.0	35.6	26.6	53.2	56.6	34.1	50.0	46.1
Short rods, Gram-variable	18.5	18.8	9.4	3.4	10.0	13.5	3.3	11.3	15.0	2.4	0.0	0.0
Short rods, changing to cocci (*Bact. globiforme* group)	12.9	9.4	11.3	8.6	10.0	6.7	10.0	8.0	7.5	14.6	0.0	7.7
Coccoid rods, Gram-positive	0.0	0.0	0.0	8.6	11.6	6.7	18.3	4.8	5.6	4.8	5.2	2.5
Cocci, Gram-positive or Gram-negative	3.7	3.7	5.6	10.3	3.3	1.7	6.6	4.8	1.9	0.0	0.0	0.0
Long rods, non-spore-forming	3.7	9.4	5.6	3.4	3.3	5.0	5.0	0.0	1.9	0.0	2.6	2.5
Rods, spore-forming	0.0	3.7	0.0	0.0	0.0	0.0	1.6	0.0	1.9	19.4	13.1	20.5

TABLE III

CHARACTERISTICS OF BACTERIAL GROUPS

(Soil N—no fertilizer)

Groups	Total no. of cult.	Per cent of total	Gr. on N. A. v. sl. or abs., %	Gelatine		NO_3 reduction, %	Soil-extr. s. s. + 1% dextrose				No action, gelatine, NO_3 or dextrose, %
				Growth, %	Liquef., %		No gr., %	Growth			
								Acid, %	Alk., %	No ch., %	
Short rods, Gram-positive	63	29.6	54.0	57.1	25.4	49.2	3.0	53.9	20.6	22.5	11.1
Short rods, Gram-negative	65	30.5	50.8	56.9	13.8	15.3	13.8	24.6	26.1	35.5	35.4
Short rods, Gram-variable	15	7.0	100.0	33.3	0.0	86.6	0.0	20.0	13.4	66.6	13.4
Short rods, changing to cocci (*Bact. globiforme* group)	24	11.2	0.0	100.0	100.0	33.3	0.0	75.0	16.6	8.4	0.0
Coccoid rods, Gram-positive	18	8.4	88.8	38.8	16.6	61.1	11.1	38.8	27.7	22.4	16.6
Cocci, Gram-positive or negative	12	5.6	91.6	41.6	33.3	66.6	8.4	41.6	25.0	25.0	0.0
Long rods, non-spore-forming	7	3.3	57.1	85.7	14.3	14.3	14.3	28.5	28.5	28.7	42.9
Rods, spore-forming	9	4.2	11.1	66.6	44.4	44.4	11.1	66.6	0.0	22.3	22.3

TABLE IV

CHARACTERISTICS OF BACTERIAL GROUPS

(Soil X—farmyard manure)

Groups	Total no. of cult.	Per cent of total	Gr. on N. A. v. sl. or abs., %	Gelatine		NO_3 reduction, %	Soil-extr. s. s. + 1% dextrose				No action, gelatine, NO_3 or dextrose, %
				Growth, %	Liquef., %		No gr., %	Growth			
								Acid, %	Alk., %	No ch., %	
Short rods, Gram-positive	65	30.5	72.3	38.5	16.9	60.0	16.9	46.1	10.8	26.2	16.9
Short rods, Gram-negative	75	35.2	49.4	40.0	17.3	12.0	17.3	25.3	24.0	33.4	38.5
Short rods, Gram-variable	23	10.8	100.0	40.0	17.4	82.5	4.3	34.8	13.0	47.9	8.7
Short rods, changing to cocci (*Bact. globiforme* group)	16	7.5	0.0	100.0	100.0	25.0	0.0	75.0	12.5	12.5	0.0
Coccoid rods, Gram-positive	12	5.6	83.3	33.3	33.3	75.0	8.3	50.0	16.7	25.0	8.3
Cocci, Gram-positive or negative	7	3.3	100.0	57.1	14.3	85.7	0.0	42.8	14.3	42.9	0.0
Long rods, non-spore-forming	8	3.8	12.5	87.5	50.0	12.5	12.5	12.5	25.0	50.0	25.0
Rods, spore-forming	7	3.2	0.0	85.7	85.7	85.7	0.0	42.8	14.2	43.0	0.0

TABLE V

CHARACTERISTICS OF BACTERIAL GROUPS

(Soil Y—mineral fertilizers)

Groups	Total no. of cult.	Per cent of total	Gr. on N. A. v. sl. or abs., %	Gelatine		NO_3 reduction, %	Soil-extr. s. s. + 1% dextrose				No action, gelatine, NO_3 or dextrose, %
				Growth, %	Liquef., %		No gr., %	Growth			
								Acid, %	Alk., %	No ch., %	
Short rods, Gram-positive	50	24.5	84.0	48.0	18.0	48.0	14.0	46.0	10.0	30.0	30.0
Short rods, Gram-negative	86	42.1	64.0	48.6	19.7	5.0	23.2	37.0	19.7	20.1	38.3
Short rods, Gram-variable	21	10.3	100.0	47.6	33.3	66.6	4.8	42.8	23.8	28.6	4.8
Short rods, changing to cocci (*Bact. globiforme* group)	17	8.3	0.0	100.0	100.0	52.9	0.0	82.3	0.0	17.7	0.0
Coccoid rods, Gram-positive	8	3.9	100.0	50.0	25.0	87.5	0.0	50.0	12.5	37.5	0.0
Cocci, Gram-positive or negative	5	2.4	80.0	40.0	20.0	20.0	20.0	0.0	20.0	80.0	60.0
Long rods, non-spore-forming	9	4.4	33.4	55.5	11.1	11.1	22.2	33.3	22.2	22.7	22.2
Rods, spore-forming	8	3.9	0.0	100.0	75.0	25.0	12.5	50.0	12.5	25.0	12.5

considered as fairly selective while there is no indication of what proportions of the organisms studied by her originated on the several media used.

Gram-positive short rods. Gram-positive short rods were the second most abundant group in all soils. This group displayed rather more activity than Gram-negative forms as judged by ability to reduce nitrates, liquefy gelatine or utilize dextrose. Like the latter group, however, a considerable percentage showed no growth on ordinary gelatine or agar and are believed to represent largely forms indigenous to soil only, brought out by such media as soil extract.

Gram-variable short rods. Gram-variable short rods appeared to comprise a definite group failing to give a uniform reaction to Gram staining, though both Hucker's and Kopeloff's modifications were used. Though numerically less important than either of the above groups they showed certain characteristics which presumably justified their being classified separately. None of 59 strains isolated was able to grow on nutrient agar while their most pronounced biochemical feature was their comparatively high nitrate-reducing ability.

Cocci-forming rods. Cocci-forming rods classified as the *Bacterium globiforme* group were an important group in all soils studied, comprising 11.2%, 7.5% and 8.3% of the organisms isolated from Soils N, X and Y respectively. Members of this group, though definite rod forms in young cultures, show a change to the coccal form with longer incubation. As previously indicated (10) variations in cell size and rate of change from rod to coccus are noted between different strains of this group. Physiological tests further emphasized differences which may be exhibited by apparently closely related strains. Thus in a separate experiment in which 50 cultures of *Bact. globiforme* were compared as to ability to utilize six sugars, hydrolyze starch and reduce nitrates, 40 actual variations in physiology were noted. As a group these organisms were the most active of those found, and were uniform in ability to liquefy gelatine and grow on standard media.

Coccoid rods. Coccoid rods, representing Gram-positive short rods that could not be satisfactorily differentiated from cocci, produced 8.4%, 5.6%, and 3.9% of the organisms isolated from Soils N, X, and Y. Like the Gram-variable short rods, the great majority failed to grow on nutrient agar, and included a large proportion of nitrate-reducing forms.

Cocci, Long Rods and Spore-formers

These three groups represent less prominent soil bacteria, judging from their abundance in the soils studied. Cocci comprised 5.6%, 3.3% and 2.4% of the strains isolated from the three soils. Conn (1, 2) found cocci to be numerically insignificant, and inclined to the belief that they are not to be regarded as characteristic of soil. In the soils studied by us the cocci isolated showed much variation in type and appeared to represent a variety of species each present in but small numbers.

Non-spore-forming long rods. This group comprised 3.3%, 3.8% and 4.4% of the total organisms isolated from the three soils, while spore-forming

rods were found to the extent of 4.2%, 3.2% and 3.9%. Only in the case of the July sampling did the last-named group represent an appreciable percentage of the forms isolated.

Discussion of Predominant Forms

A close comparison with the short rod forms described by Conn (2, 3) and Topping (13) is made difficult on account of the use of different media, making for differences in grouping and in estimating relative abundance in soil. Thus Gram-negative rods were of greater significance than is indicated by the work of these authors. However it appears that the non-spore-forming short rods comprising our Groups I to V correspond in large measure to types of short rods described by Conn and particularly to those classed as "slow growers" or "punctiform colony forming" organisms. Such terms may be misleading in some instances, since organisms producing little growth on a comparatively deficient medium, such as tap-water gelatine, may grow profusely on other media. This has been found to be the case with organisms included in our *Bact. globiforme* Group IV.

It appears that Conn's Group I, comprising simple rods, includes types which we have subdivided into our Groups I, II, III, and V. On the other hand our *Bact. globiforme* group is rather more inclusive than Conn's, embracing not only his Group II *(Bact. globiforme)*, comprising forms showing change from rod to coccus, but also his less abundant Groups III and IV, characterized by a tendency to produce branched forms. The last-named groups are doubtless related to pleomorphic, coccus-forming types forming "sprouts", or branching forms described by Topping, who suggests a close relationship between her Groups 1 and 2 and *Bact. globiforme*. Belief in this relationship is strengthened by observation of strains of *Bact. globiforme* including one obtained from Dr. Conn which show ability, especially in liquid media, to produce branching forms characteristic of Conn's Groups III and IV and Topping's pleomorphic groups. Moreover comparison of Figs. 11, 5, 3 and 15 given in Topping's paper with Figs. 1, 2, 3 and 5 respectively in the paper of Taylor and Lochhead (10) on *Bact. globiforme* suggests that these authors worked with very closely related forms. The apparently higher incidence of pleomorphic types comprising *Bact. globiforme* and related forms found by Conn and Topping as compared with our present studies is believed to be due largely to the use of media more selective for these forms. The selective action of tap-water gelatine as compared with soil extract agar has been previously demonstrated (10).

Relation of Bacterial Groups to Soils Studied

In Table II, giving the incidence of the different morphological groups in the three soils at four sampling dates, no outstanding differences are seen between the unfertilized soil, N, and the fertilized soils, X and Y, in spite of great variation in crop-producing capacity. The uniformity is the more interesting since Soil X received an application of farmyard manure three

weeks previous to the November sampling. Slightly higher percentages of non-spore-forming long rods and of spore-formers were noted in this soil but otherwise the group incidence approximated closely that of Soils N and Y. In line with the findings of Joffe and Conn (4) it is apparent that if notable increases in spore-formers are to result from addition of organic materials, amounts in excess of normal field applications are needed.

Throughout the tests there was no indication that the incidence of *Bact. globiforme* bore any relationship to the productivity of the soils examined, nor was it possible to correlate fertility with the relative abundance of any of the other main groups into which the organisms were classified. The soils in question, originally the same, had become different in crop-producing ability by artificial means, and the results suggest that in a soil of given type the bacterial flora may be fairly resistant to change, even though productivity may vary as much as ten-fold.

There is indication of rather more difference in group incidence due to cropping than between the soils themselves. Comparative data, following timothy and mangels respectively, are given in Table VI, in which the approximate numbers in millions per gram dry soil are shown. After mangels, increased counts of Gram-positive and Gram-variable short rods and *Bact. globiforme* were found, as compared with the numbers following timothy. Mangels in general supported a higher bacterial population as measured by both total and plate counts (Table I).

TABLE VI

BACTERIAL GROUPS IN THREE SOILS FOLLOWING DIFFERENT CROPS

Morphological group	Millions per gram of dry soil					
	Following timothy			Following mangels		
	N	X	Y	N	X	Y
Short rods, Gram-positive	20.0	22.3	34.6	28.7	55.7	44.1
Short rods, Gram-negative	30.8	20.9	30.9	16.4	23.2	27.3
Short rods, Gram-variable	15.4	14.9	9.1	24.6	16.2	14.7
Short rods, changing to cocci (*Bact. globiforme* group)	10.7	7.4	9.1	18.4	13.9	14.7
Cocci, Gram-pos. and Gram-neg.	3.1	3.0	5.5	8.2	0.0	12.6
Long rods, non-spore-forming	3.1	7.4	5.5	8.2	6.9	2.1
Rods, spore-forming	0.0	3.0	0.0	4.2	0 0	0.0

The effect of season on the relative incidence of the different bacterial groups was in general not marked (Table II). The most pronounced changes occurred in the July sampling, which showed a decrease in Gram-variable short rods and a rather notable increase in spore-formers in all soils. At this sampling the mangel crop was growing but no definite reason for the degree of prominence of this latter group is offered.

Conclusion

Micro-organisms in the untreated soil, N, might be expected to represent for the most part the *autochthonous* microflora, *i.e.*, the indigenous group of

organisms postulated by Winogradsky (14, 15) as contrasted with the *zymogenic* group, composed of forms relatively scarce in normal soils, but becoming active upon additions of readily decomposable substances. This latter group might be supposed to be more evident in Soil X, receiving farmyard manure. As judged from the grouping of the bacteria isolated, however, no evidence of alteration of types was found, suggesting that the autochthonous flora of the soil type studied was little affected by the treatments given. Whether differences exist that are not brought out by general grouping, would have to be decided by studies of a much more specific nature.

More detailed study of strains isolated, however, particularly physiological tests, showed a surprising variability in apparently very closely related types. In each of the main groups identity of characteristics was the exception rather than the rule, with each additional test bringing out slight divergencies. The degree of biochemical activity displayed by the predominant soil organisms was regarded as relatively low. It is suggested that the indigenous bacterial flora is comparatively unstable physiologically and possessed of considerable adaptability. The comparative inactivity of so many forms when isolated from soil and cultivated singly also suggests that the functions of these species are exercised most fully only when they are acting in association with other micro-organisms. In aiding toward a better understanding of these functions research with mixed cultures will doubtless play an important part. The limitations attendant on an investigation of one type of soil, as in the present study, are recognized, and it is hoped to extend the work to a variety of fertile and infertile soils of different types.

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QUALITATIVE STUDIES OF SOIL MICRO-ORGANISMS

III. INFLUENCE OF PLANT GROWTH ON THE CHARACTER OF THE BACTERIAL FLORA[1]

By A. G. Lochhead[2]

Abstract

Comparative studies of the relative incidence of bacterial types occurring in the rhizosphere of different plants and in control soils indicated that the qualitative nature of the soil microflora is markedly influenced by the growing plant. In the rhizosphere Gram-negative rods are proportionately increased while Gram-positive rods, coccoid rods, and spore-forming types are relatively less abundant.

The majority of bacteria isolated from soil by non-selective plating are forms included in the family Proactinomycetaceae (Jensen's classification). Of these by far the largest group consists of members of the genus *Corynebacterium*. In the rhizosphere proactinomycetes as a whole are relatively less abundant, with the *Corynebacterium* (non-motile) group likewise depressed. However, closely related motile forms classed as *Mycoplana* are preferentially stimulated.

In the rhizosphere the bacteria show definitely greater physiological activity than in soil distant from the plant. Not only is there a notably greater proportion of motile forms, and a pronounced increase in the incidence of chromogenic types, but also a higher incidence of liquefying bacteria and of those able to affect glucose.

A comparison of the rhizosphere of certain plant varieties resistant and susceptible respectively to soil-borne disease showed differences of a qualitative nature in the bacterial flora suggestive of a greater "rhizosphere effect" in the case of the susceptible varieties studied. Results point to the possibility that resistance may be associated with a selective action of root excretions on the saprophytic soil microflora.

Introduction

In a previous paper of this series (16), following a general introduction (9), a report was made of investigations carried out on a non-selective basis of the qualitative nature and relative incidence of the bacterial types occurring in soils differing in fertility. On the basis of morphology in soil extract semi-solid medium, in which the organisms were considered to correspond more closely to their form in soil than when cultivated on more "synthetic" substrates, the bacteria were classified into eight groups. In spite of unequal productivity the soils showed no outstanding differences in the relative abundance of the various morphological groups, while grouping on the basis of physiological activity likewise showed a surprising degree of uniformity in the soils studied. A brief summary of the characters of the organisms found is given in Table I, recalculated from previous data for comparison with findings as to the effect of plant growth presented below.

The results suggested that the character of the *autochthonous* (indigenous) soil microflora is relatively uniform in soil of definite type, and comparatively

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little affected by normal field application of fertilizers resulting in greatly altered crop-producing capacity. The predominant soil bacteria appeared relatively inactive in single culture, with considerable divergence in biochemical action shown by apparently closely related forms. The findings suggested that the bacterial flora of soil is unstable physiologically, with considerable adaptability, and that the functions of the different types are exercised most fully only under conditions of association.

The object of the present investigation was to study the effect of the growing plant upon the nature of the soil bacteria, and compare the characters of bacteria in the rhizosphere with those of organisms away from the zone of influence of the plant.

The Rhizosphere—Qualitative Aspects

Some 35 years ago Hiltner (1) observed that certain micro-organisms showed increased activity close to the roots of cultivated plants and called this zone influenced by root excretions the "rhizosphere". Since then various other investigators have confirmed his findings, and shown that soil in the vicinity of the roots supports usually noticeably higher numbers of micro-organisms than soil more distant from the plant. Purely quantitative considerations, however, need not concern us here.

Our information as to the effect of plant growth on the qualitative nature of soil bacteria is meagre, and confined largely to data on the numbers of organisms appearing on selective media chosen to favour various groups. Thus Starkey (13, 14) found that the development of higher plants exerted greater influences upon some groups of organisms than upon others. Whereas the proportionate increases in nitrogen-fixing organisms, actinomycetes, and filamentous fungi were slight, organisms of the *B. radiobacter* group were preferentially stimulated in the rhizosphere. It was found, however, that different plants exerted different degrees of influence upon soil micro-organisms.

Krassilnikov (6) reported that *Azotobacter* was unable to grow in the rhizosphere of wheat and was severely repressed in that of maize, and attributes this to the toxic effect of root secretions. Similarly *B. mycoides*, *B. megatherium*, and yeasts of the type of *S. cerevisiae* were unable to grow in the rhizosphere of the same plants. On the other hand, small non-sporing bacteria of the types of *B. denitrificans* and *B. fluorescens* were found to multiply intensively in the rhizosphere, presumably under the stimulation of organic root excretions.

Evidence of a selective action exerted by the root system on soil bacteria was obtained by Krassilnikov, Kriss, and Litvinov (7), who reported striking increases in cellulose-decomposing organisms and of small non-spore-forming rods growing poorly under laboratory conditions. The function of this latter group of bacteria, which are characterized by ability to reduce nitrates to nitrites, is unknown. The same authors in a later paper (8) found the predominant organisms in the rhizosphere to consist of non-sporing rods with

well pronounced ammonifying capacity, and of mycobacteria. Spore-formers comprised an insignificant proportion of the rhizosphere population, though some rise was apparent towards the end of the vegetation period of the plants.

Further information as to predominant bacterial types developing about roots of growing plants has been given by Starkey (15) from microscopic observations of the rhizosphere. Employing the buried slide technique, he observed that the bacteria occurring in greatest abundance about root hairs, fungus filaments, and decomposing organic matter were small, coccoid cells. Longer rods were detected, but spore-formers were seldom encountered.

Studying the effect of organic soil amendments on the microflora of wheat roots, Thom, Clark, Fierke, and Fellows (17) found corynebacteria to be the most numerous types when either chicken manure or chopped green alfalfa was added to soil, though certain differences in physiological properties of the predominant organisms were observed under the two conditions. The observations were concerned with the influence of soil treatment rather than with the effect of the plant in modifying the soil microflora.

Experimental

The present report embraces qualitative studies of the bacterial flora of the rhizosphere of red clover, mangels, oats, tobacco, corn, and flax to note changes exerted by the various crops on the relative incidence of types present in control soils. In addition, comparative studies were made of the rhizosphere of certain varieties of tobacco and flax resistant and susceptible respectively to soil-borne fungous diseases.

For the analysis of field samples, blocks of soil about the plants were carefully dug up and brought to the laboratory. After removal of above-ground portions of the plants, the soil was gently broken to release the root system, which was added to sterile water together with adherent soil after gentle shaking to remove superfluous quantities. After thorough shaking suitable dilutions were prepared from the original flask for plating. The roots were later removed, contents of flask measured and evaporated to dryness to permit of the estimation of plate counts of rhizosphere soil on a dry weight basis. Controls consisted of corresponding composite soil samples distant from the plant; in the case of different plant varieties, e.g., tobacco, the rhizospheres of which were being compared, midway between rows. In the case of the greenhouse samples, plants were carefully removed from the pots and similarly treated. Controls in this case consisted of the remainder of the soil in the pot after thorough mixing.

Examination of rhizosphere and control soil was made on a quanti-qualitative basis following general procedures previously described (16). This involved a study of colonies growing on soil extract agar prepared according to Löhnis (10) without added energy material, and chosen as being as non-selective as possible, and hence most desirable for primary isolation purposes. While a "richer" medium would allow better colony development of many forms, this was considered a distinct disadvantage in view of the antagonisms

possible in plate cultures leading to the suppression of other forms which might otherwise appear, even as small colonies.

From representative plates, 60 to 100 colonies were picked, representing all on a plate or a sector, and stab cultures made to soil extract semi-solid medium (containing 0.02% K_2HPO_4, 0.01% yeast extract and 0.3% agar). The use of this medium assured the survival of 93% of the transfers, many from pin-point colonies. Obvious actinomycetes were not included. The present report is based on data from a study of 1,191 cultures.

Morphological observations of predominant soil types had indicated the presence of a large proportion of pleomorphic organisms, which in the majority of cases showed affinity to the corynebacteria. It was also observed that whereas on the richer artificial media pleomorphism was quite pronounced, little or none was observed in sterilized soil, or in soil extract semi-solid medium. Classification of the strains isolated, therefore, was made (i) on the basis of the forms occurring in soil extract semi-solid medium and considered to represent more closely the morphology of the organisms in soil, and (ii) on the basis of additional observations on a variety of richer artificial media to differentiate forms with a view to noting more closely their taxonomic relationship. For this purpose nutrient agar and yeast peptone agar as employed by Topping (19, 20) in her soil flora studies were used. Tests for motility, staining reactions, and biochemical properties were included to aid in grouping.

Comparison of Rhizosphere and Control Soils

In Table II is presented a summary of morphological groups as observed in soil extract semi-solid medium, in addition to certain physiological groups, derived from a study of individual cultures from the rhizosphere of various cultivated plants. In contrast to Table I, which indicates little or no difference due to fertilizer treatment, Table II reveals certain striking effects exerted by the growing plant on the bacterial groups in the soil.

While different plants show variations, both in total numbers and in the incidence of different microbial groups, yet there is noted an unmistakable selective action characteristic of the rhizosphere of all plants studied. In all cases Gram-negative short rods are proportionately increased in the rhizosphere as compared with the control soil. On the other hand Gram-positive short rods, coccoid rods, and spore-forming bacteria are relatively less numerous in the rhizosphere than in soil more distant from the plant. In the case of Gram-variable rods, *Bact. globiforme* group, cocci, and long non-sporing rods, no definite rhizosphere effect is noted suggestive of group stimulation or depression due to plant growth.

From the standpoint of bacterial physiology, the findings indicate a more active bacterial flora in the rhizosphere than in the soil beyond the zone of influence of the plant. In the rhizosphere is found a higher percentage of forms developing well on nutrient agar, of liquefying types, and of those causing acid or alkaline reactions in dextrose. A noticeably greater percentage

TABLE I

COMPARISON OF BACTERIAL GROUPS IN SOILS OF DIFFERENT FERTILITY

	Soil N (no fertilizer)	Soil X (farmyard manure)	Soil Y (mineral fertilizer)
Relative crop producing capacity			
Timothy	55.9	100.0	85.1
Mangels	8.9	100.0	86.5
Morphological groups (soil extract)	%	%	%
Short rods, Gram-pos.	29.6	30.5	24.5
Short rods, Gram-neg.	30.5	35.2	42.1
Short rods, Gram-var.	7.0	10.8	10.3
Bact. globiforme group	11.2	7.5	8.3
Coccoid rods, Gram-pos.	8.4	5.6	3.9
Cocci	5.6	3.3	2.4
Long rods, non-sporing	3.3	3.8	4.4
Spore-formers	4.2	3.2	3.9
Physiological groups	%	%	%
Growth on N. A. very slight or absent	53.5	58.7	66.1
Gelatin liquefaction	28.6	27.7	29.4
Nitrate reduction	40.4	43.7	30.4
Acid reaction in dextrose 1%	42.7	38.5	43.6
Alkaline reaction in dextrose 1%	21.6	16.9	15.7

of motile organisms is present in the rhizosphere, while the incidence of chromogenic types shows a very pronounced increase over that in the control soils.

The more detailed comparison of the tobacco, corn, and flax soils, based on more extended observation to note specially pleomorphic forms, is summarized in Table III. Further points of interest are brought out in the relative incidence of certain broad generic groups as affected by plant growth. Non-sporing, non-pleomorphic, rod-shaped bacteria are proportionately increased in the rhizosphere, particularly Gram-negative forms. On the other hand, the broad group of pleomorphic organisms, comprising well over half the forms in the control soils, are relatively less abundant.

Investigations of such workers as Jensen (2–4), Ørskov (11, 12), Krassilnikov (5), Topping (19), and Umbreit (21) have called attention to the occurrence in soil of appreciable numbers of organisms belonging to non-sporing genera of the order Actinomycetales, forms showing characteristically the properties of bacteria, though usually with a considerable degree of pleomorphism and in some cases with a tendency towards formation of slight mycelium. The taxonomy of the order is at present confused and the nomenclature for what are apparently similar groups most varied. The classification of soil forms is hindered, not only by lack of sufficient study, but also because many soil species do not appear to fit into schemes of classification based largely on the more "classical" parasitic forms.

TABLE II

INCIDENCE OF BACTERIAL GROUPS IN RHIZOSPHERE AND CONTROL SOILS (A)

Classification	Rotation plots (N)				Tobacco soil			Corn soil		Flax (pot expt.)		
	Control	Rhizosphere			Control	Rhizosphere		Control	Rhizo.	Control	Rhizosphere	
		Clover	Mangels	Oats		RH. 211	CH. 38				Bison	Novelty
Plate count (millions)	48.6	254.8	108.2	118.1	94.7	269.2	505.4	28.2	389.2	98.3	439.9	2751.3
Morphology, Soil ex. semi-solid	%	%	%	%	%	%	%	%	%	%	%	%
Short rods, Gram-pos.	19.7	8.7	10.8	13.6	23.3	20.0	16.1	23.1	22.2	7.7	6.1	1.4
Short rods, Gram-neg.	30.3	55.1	43.1	45.5	13.3	43.3	45.2	26.9	51.1	15.4	36.4	59.1
Short rods, Gram-var.	16.7	15.9	15.4	19.7	11.7	6.7	17.7	5.8	6.7	7.7	7.1	14.1
Bact. globiforme group	7.6	1.5	10.8	1.5	13.3	10.0	16.1	7.7	4.4	1.9	16.2	1.4
Coccoid rods, Gram-pos.	13.6	5.8	10.8	10.6	18.3	10.0	4.8	21.2	13.3	53.8	32.3	16.9
Cocci	1.5	1.5	3.1	3.0	1.7	3.3	0.0	0.0	0.0	7.7	0.0	1.4
Long rods, non-sporing	4.6	8.7	6.2	3.0	6.7	5.0	0.0	5.8	2.2	0.0	1.0	5.6
Spore-formers	6.1	2.9	0.0	3.0	10.0	1.7	0.0	9.6	0.0	5.8	1.0	0.0
Physiology												
Good growth, nutrient agar	33.3	56.5	61.5	45.5	35.0	75.0	66.1	48.1	57.7	36.5	78.8	85.9
Chromogenic types	12.1	29.0	29.2	21.2	1.7	26.7	29.0	3.8	33.3	7.7	29.3	25.4
Motile forms	—	—	—	—	13.3	31.7	25.8	13.5	24.4	26.9	36.4	64.8
Liquefying (1 wk.)	—	—	—	—	15.0	16.7	41.9	23.1	24.4	42.3	54.6	70.4
Inhibited, 1% dextrose	42.4	27.5	20.0	22.7	30.0	25.0	25.8	11.5	8.9	5.8	1.0	0.0
Acid reaction, dextrose	39.4	42.1	53.8	43.9	36.7	38.3	48.4	56.7	57.8	50.0	67.7	81.7
Alk. reaction, dextrose	6.1	15.9	16.9	21.2	16.7	21.7	17.7	9.6	17.8	3.9	9.1	12.7

TABLE III

INCIDENCE OF BACTERIAL GROUPS IN RHIZOSPHERE AND CONTROL SOILS (B)

Classification	Tobacco soil			Corn soil		Flax (pot expt.)		
	Control	Rhizosphere		Control	Rhizo.	Control	Rhizosphere	
		RH. 211	CH. 38				Bison	Novelty
Plate count (millions)	94.7	269.2	505.4	28.2	389.2	98.3	439.9	2751.3
Non-pleomorphic	%	%	%	%	%	%	%	%
Cocci	1.7	1.7	0.0	0.0	0.0	1.9	0.0	0.0
Rods, spore-forming	10.0	1.7	0.0	9.6	0.0	5.8	1.0	0.0
Rods, non-sporing, total	21.6	41.7	40.3	28.8	44.4	11.5	24.2	54.9
Rods, non-sporing, Gram-pos.	18.3	20.0	20.9	21.2	24.4	5.8	10.1	18.3
Rods, non-sporing, Gram-neg.	3.3	21.7	19.4	7.5	20.0	5.8	14.1	36.6
Rods, non-sporing (pct. Gram-pos.)	(84.6)	(45.8)	(52.0)	(73.3)	(55.0)	(50.0)	(41.7)	(33.3)
Rods, non-sporing (pct. motile)	(38.5)	(48.0)	(41.7)	(6.7)	(30.0)	(33.3)	(50.0)	(66.7)
Rods, non-sporing (pct. chromogenic)	(0.0)	(32.0)	(28.0)	(13.3)	(53.0)	(0.0)	(41.7)	(26.2)
Pleomorphic								
Total (Proactinomycetaceae)	65.0	55.0	58.1	53.9	48.9	80.7	74.5	45.1
Acid-fast, non-motile, Gram-pos. (*Mycobacterium*)	8.3	0.0	1.6	1.9	2.2	3.8	0.0	0.0
Non-acid-fast, non-motile (*Corynebacterium*)								
Total	55.0	45.0	45.2	42.3	35.6	57.7	50.5	16.9
Gram-pos.	43.3	33.3	38.7	40.4	24.4	44.2	42.4	8.5
Gram-neg.	11.7	11.7	6.5	1.9	11.1	13.5	8.1	8.5
Non-acid-fast, motile (*Mycoplana*)								
Total	1.7	10.0	11.3	9.6	11.1	19.2	24.2	28.2
Gram-pos.	1.7	1.7	6.5	9.6	8.9	13.5	13.1	5.6
Gram-neg.	0.0	8.3	4.8	0.0	2.2	5.8	11.1	22.6
Non-acid fast (pct. Gram-pos.)	(81.9)	(63.7)	(80.0)	(96.3)	(71.4)	(75.0)	(74.3)	(35.5)
Non-acid-fast (pct. motile)	(2.9)	(18.2)	(20.0)	(18.4)	(23.8)	(25.0)	(32.4)	(61.3)
Non-acid-fast (pct. chromogenic)	(2.9)	(24.2)	(31.4)	(0.0)	(19.0)	(10.0)	(24.3)	(22.6)
Non-acid-fast (pct. liquefy.)	(14.7)	(24.2)	(54.3)	(22.2)	(23.8)	(52.5)	(56.8)	(54.8)
Non-acid-fast (pct. reduce NO_3)	(47.1)	(30.3)	(42.8)	(22.2)	(18.0)	(50.0)	(14.9)	(41.9)

Pleomorphic types considered to fall within the family Proactinomycetaceae following Jensen's (2) classification of the Actinomycetales, were found to comprise a large proportion of the bacteria in the control soils. It was found that clear-cut distinction of strains on the basis of such properties as slight mycelium formation, acid fastness, or reaction to the Gram stain is not entirely satisfactory in the separation of forms occurring in soil. Jensen (2) has already suggested that the transition from *Proactinomyces*, the criterion for which genus is the formation of initial mycelium, to *Mycobacterium* and *Corynebacterium* is very gradual, while Topping (19), Ørskov (12), and Umbreit (21) have also stressed the difficulty of definite distinction between these groups on the basis of slight mycelium formation. In the soils studied, mycelium formation was observed with relatively few strains and in the absence of other pronounced features distinguishing this group, they were classified, for the present purpose, with the acid-fast group (mycobacteria) or the non-acid-fast group (corynebacteria).

Non-acid-fast forms comprised the overwhelming majority of the proactinomycetes in the soils examined. These organisms exhibited characteristic "snapping" division in the rod stage and showed a tendency, particularly on richer artificial media, towards the production of irregular, swollen forms, often slightly branched or showing formation of buds or "sprouts". In older cultures, and more especially in soil extract media, the organisms appeared more predominantly as coccoids, arising from a process of fragmentation. Comparison of these forms, which showed closest affinity to the genus *Corynebacterium*, indicated that many apparently closely related strains differed in their reaction to the Gram stain and with respect to motility. Since the generic term *Corynebacterium* is usually reserved for non-acid-fast, non-motile, Gram-positive forms, the taxonomy of motile or Gram-negative forms may well be a matter of dispute. Some provision appears necessary for motile soil forms related to corynebacteria, the presence of which has been also stressed by Topping (19) and Ørskov (12). For the present purpose, non-motile forms are classified as *Corynebacterium*, with recognition of both Gram-positive and Gram-negative types, while motile forms are considered to show closest affinity to the genus *Mycoplana* Gray and Thornton, to include likewise both Gram-positive and Gram-negative forms. Whether this separation of motile from non-motile forms is preferable to the inclusion of both forms in the genus *Corynebacterium* will doubtless await further investigation, though some support of the latter alternative is given by our finding of occasional motile strains of the *Bacterium globiforme* group. The characteristic *Bact. globiforme* is now believed by us to represent a special group of the corynebacteria with distinctive cultural and physiological properties. In the present comparison of control soils and rhizosphere, where formal classification and identification of species were regarded as less important than the grouping of organisms according to certain morphological and physiological characters, it was found of advantage to consider motility and Gram reaction in differentiating between strains.

As noted from Table III the relative incidence of proactinomycetes as a whole is lower in the rhizosphere than in the control soils. Within the broad group, however, a selective action by the plant is apparent. The largest group in the control soils consists of non-acid-fast, non-motile forms regarded as corynebacteria. This group appears to correspond to organisms found by Jensen (3) to comprise an important group of organisms in Australian soils, and are doubtless to be included with the "mycobacteria" reported by Krassilnikov (5) to be abundant in Russian soils. This group showed a relative depression in the rhizosphere. On the other hand, motile forms, provisionally grouped as *Mycoplana*, appeared to be preferentially stimulated in the rhizosphere of all plants studied.

With pleomorphic as well as non-pleomorphic bacteria, Gram-positive forms were relatively less numerous in the rhizosphere than in controls, while chromogenic forms were conspicuously more abundant in the rhizosphere. Proactinomycetes with liquefying capacity were rather more abundant in the rhizosphere, though nitrate-reducing forms appeared to be slightly less numerous relatively as compared with the control soils.

Rhizosphere of Resistant and Susceptible Plants

In comparative quantitative studies of the rhizosphere of certain varieties resistant and susceptible respectively to soil-borne pathogens, Timonin (18), working in this laboratory, noted higher numbers of bacteria, and to a less extent, of fungi, in the case of the susceptible than of resistant varieties. This was observed in both greenhouse and field tests with flax susceptible to wilt (*Fusarium lini*), and with tobacco susceptible to black root rot (*Thielaviopsis basicola*). With actinomycetes no difference was observed. Since the tests were made with uninfected plants, the differences observed were believed to be due to inherent differences in physiological function, making in the case of susceptible plants, conditions somewhat more favourable for general bacterial development.

Qualitative surveys of the bacterial flora of the rhizosphere which included comparisons of resistant (Bison) and susceptible (Novelty) flax, and resistant (RH. 211) and susceptible (CH. 38) tobacco, are partly summarized in Table IV. Morphological classification according to forms developing in soil extract semi-solid medium showed differences in the relative incidence of three groups of organisms. Gram-negative short rods were relatively more abundant, and coccoid rods and spore-formers less abundant, in the rhizosphere of the susceptible than of the resistant varieties of plants. As noted above (Table II) the same trend was observed with respect to rhizosphere and control soil. These findings, taken in conjunction with the quantitative data, suggest that the susceptible plants studied exerted a greater "rhizosphere effect" than did corresponding resistant varieties.

The more detailed differentiation showed few significant differences. Non-motile, non-pleomorphic short rods, and motile, pleomorphic rods (*Mycoplana?*) showed a slightly higher incidence in the rhizosphere of susceptible plants.

TABLE IV

BACTERIA IN RHIZOSPHERE OF RESISTANT AND SUSCEPTIBLE PLANTS

Classification	Flax (field)		Flax (pots)		Tobacco (field)	
	Bison (R)	Novelty (S)	Bison (R)	Novelty (S)	RH. 211 (R)	CH. 38 (S)
Plate count (millions)	13.1	18.7	439.9	2751.3	269.2	505.4
Morphology—Soil ex. semi-solid	%	%	%	%	%	%
Gram-neg. short rods	32.2	44.4	36.4	59.1	43.3	45.2
Coccoid rods, Gram-pos.	10.2	6.3	32.3	16.9	10.0	4.8
Spore-formers	16.9	4.8	1.0	0.0	1.7	0.0
Detailed differentiation (non-sporing types)						
Non-pleomorphic						
Cocci	1.7	0.0	0.0	0.0	1.7	0.0
Rods, non-sporing, total	22.1	23.9	24.2	54.9	41.7	40.3
Rods, non-sporing, Gram-pos.	5.1	4.8	10.1	18.3	20.0	20.9
Rods, non-sporing, Gram-neg.	17.1	19.1	14.1	36.6	21.7	19.5
Rods, non-sporing, motile	6.8	6.3	12.1	36.6	20.0	16.1
Rods, non-sporing, non-motile	15.3	17.6	12.1	18.3	21.7	24.2
Pleomorphic						
Acid-fast, non-motile, Gram-pos.	1.7	7.9	0.0	0.0	0.0	1.6
Non-acid-fast, non-motile, total	39.0	46.0	50.5	16.9	45.0	45.2
Non-acid-fast, non-motile, Gram-pos	23.4	28.6	42.4	8.5	33.3	38.7
Non-acid-fast, non-motile, Gram-neg.	13.6	17.4	8.1	8.5	11.7	6.5
Non-acid-fast, motile, total	13.5	14.3	24.2	28.2	10.0	11.3
Non-acid-fast, motile, Gram-pos.	8.4	9.5	13.1	5.6	1.7	6.5
Non-acid-fast, motile, Gram-neg.	5.0	4.8	11.1	22.6	8.3	4.8
Physiological						
Motile organisms	28.8	30.1	36.4	64.5	31.7	25.8
Chromogenic forms	5.1	6.3	29.3	25.4	26.7	29.0
Liquefying	15.3	15.9	54.6	70.4	16.7	41.9
Acid reaction, dextrose	45.7	46.0	67.7	81.7	38.3	48.4
Nitrate-reducing	30.5	31.7	21.2	49.3	35.0	41.9

Physiological tests pointed, if anything, to a somewhat more active microflora in the rhizosphere of susceptible plants.

Although these preliminary findings by no means prove, yet they suggest the possibility, that resistance to certain disease may be linked up with a selective action of root excretions upon the saprophytic soil microflora, thus favouring types which may be more, and in other cases less, antagonistic (directly or indirectly) towards pathogenic organisms.

Conclusion

The results as a whole emphasize the importance of enlarging our knowledge of the qualitative nature of the soil micro-organisms and particularly those types the functions of which are still unknown, but which appear to comprise a large proportion of the micro-population of arable soils. Up to the present

the study of processes, and incidentally that of specialized groups of organisms concerned in these processes, has received more attention than the objective study of soil organisms on a non-selective basis. It is believed, however, that more study from the qualitative side is essential to help complete our knowledge of the types indigenous in soil and the changes in the trend of population in the rhizosphere. It is here where not only the main interaction occurs between soil micro-organisms and the growing plant, but where the action of various soil-borne pathogens or toxic factors occurs to complicate and accentuate associative or antagonistic influences.

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QUALITATIVE STUDIES OF SOIL MICRO-ORGANISMS

IV. THE RHIZOSPHERE IN RELATION TO THE NUTRITIVE REQUIREMENTS OF SOIL BACTERIA[1]

By P. M. West[2] and A. G. Lochhead[3]

Abstract

Bacteria of the rhizospheres of flax and tobacco were found to possess more complex nutritive requirements than those in the corresponding control soils. The roots of even young seedlings favour the development of those types that are dependent upon a supply of thiamin, biotin, and amino nitrogen for their growth, thus suggesting that the roots may excrete significant amounts of these stimulative substances. The "rhizosphere effect" was more pronounced with susceptible than with resistant varieties of either flax or tobacco. A greater difference was found to exist between the rhizosphere and the control soil where the latter is poor than where it is richly supplied with organic matter, since liberation of growth substances by plant decomposition permits a limited development of the more typically rhizosphere forms, apart from the zone of influence of the growing plant.

Introduction

On the basis of cell morphology and taxonomic relationship, Lochhead (2) found qualitative differences between bacteria in the rhizosphere and in soil distant from plant roots. It was observed also that the growing plant appeared to be a greater factor in determining the relative predominance of different morphological types of micro-organisms in soil of definite type than did various fertilizer treatments, which, as had been shown by Taylor and Lochhead (3), exerted relatively little effect on the incidence of the various groups of bacteria. These results suggested that the plant, through the agency of root excretions, might play an important role in the nutrition of rhizosphere forms, and that in addition to differences in the relative occurrence of various morphological types in the rhizosphere compared to normal soil, there might also be significant differences in the nutritive requirements of the organisms in the two groups. This possibility was investigated, since the existence of any specific nutritive differences would not only give some indication concerning the nature of the heretofore hypothetical "plant excretions", but also explain, at least in part, the mechanism by which the plant induces the development of the characteristic rhizosphere flora.

Experimental

From the rhizospheres of Bison and Novelty flax, resistant and susceptible respectively to wilt, 100 representative bacteria were isolated according to quanti-qualitative methods previously described (2, 3). The plants were grown under greenhouse conditions in pots containing uniform soil. One hundred control organisms were isolated from the pot soil after the roots, and

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the soil adherent to them, had been removed. These organisms were inoculated into four media of increasing complexity.

(1) Base medium consisting of glucose 1.0 gm., K_2HPO_4 1.0 gm., KNO_3 0.5 gm., $MgSO_4$ 0.2 gm., $CaCl_2$ 0.1 gm., NaCl 0.1 gm., $FeCl_3$ 0.01 gm., distilled water, 1 litre. Reaction, pH 6.8.

(2) Base medium enriched with amino nitrogen: asparagine 0.5 gm., aspartic acid 0.1 gm., cysteine 0.1 gm., glycine 0.1 gm., alanine 0.1 gm. per litre.

(3) Base medium enriched with amino nitrogen, (as above) together with a combination of bacterial growth factors: thiamin (vitamin B_1) 0.1γ, biotin 0.0005γ, β-alanine 0.1γ, nicotinic acid 0.1γ, and i-inositol 0.1 mg. per ml.

(4) Base medium enriched with yeast extract 1.0 gm. per litre.

All amino acids and growth factors were used in pure form with the exception of biotin, available as a concentrate prepared by the procedure of Kögl and Tonnis (1).

The complexity of the nutritive requirements of the bacteria from rhizosphere and control soils was determined by their growth responses in the above media after five days' incubation at 28° C. The simplest requirements were shown by those organisms producing heavy growth in the base medium, and growing no more abundantly in the media to which supplements had been added. Others, while capable of development at a sub-maximal rate in the base medium, grew more rapidly on one or more of the other substrates. The most fastidious organisms were quite unable to grow in the base medium, and in these cases the presence of amino nitrogen or growth factors was absolutely essential.

Requirements of Bacteria from Flax Rhizosphere

As shown in Table I and Fig. 1, in comparison to the controls, organisms from the rhizosphere of Bison (resistant) showed an 83% proportionate increase in numbers for which amino nitrogen was either stimulative or essential, while the organisms from Novelty (susceptible) showed a 325% increase. The numbers of bacteria influenced by the combined growth factors were increased 71% in the rhizosphere of Bison, and 143% in the rhizosphere of Novelty, beyond their incidence in the control soil. These findings indicate that in addition to the morphological differences observed (2) between the predominant types in rhizosphere and control soils, there also occurs a marked difference in the nutritive requirements of the organisms in the two groups. The greater proportion of the bacteria in the flax rhizosphere appear to depend on a supply of certain amino acids or growth substances for their maximum development, while the majority of the organisms in the check soils are independent of these factors for their growth. Furthermore, it is apparent that the relative incidence of various nutritional forms differs more widely from the control soil in the rhizosphere of the susceptible flax variety than in the rhizosphere of the resistant.

TABLE I

RELATIVE INCIDENCE OF BACTERIA OF VARIOUS NUTRITIVE TYPES IN RHIZOSPHERE OF FLAX AND TOBACCO AND IN CONTROL SOILS

	Flax			Tobacco		
	Control	Rhizosphere		Control	Rhizosphere	
		Res.	Susc.		Res.	Susc.
	%	%	%	%	%	%
Growth in base medium	2.1	49.4	39.7	43.0	36.0	50.0
Amino acids essential for growth	14.7	16.9	44.1	11.8	19.5	29.0
Amino acids stimulative	0.0	10.4	19.1	6.4	13.4	22.0
Either essential or stimulative	14.7	27.3	63.2	18.2	32.9	51.0
Growth factors essential	25.6	26.4	48.2	24.7	34.0	41.8
Growth factors stimulative	4.0	24.3	23.8	12.7	14.4	19.7
Either essential or stimulative	29.6	50.7	72.0	37.4	48.4	61.5
Stimulated by yeast extract only	67.8	19.5	14.7	26.7	26.8	6.9
No stimulation in any medium	2.1	30.0	13.2	34.2	22.6	25.5
Amino acids and growth factors without effect	69.9	49.5	28.0	61.0	49.4	32.4

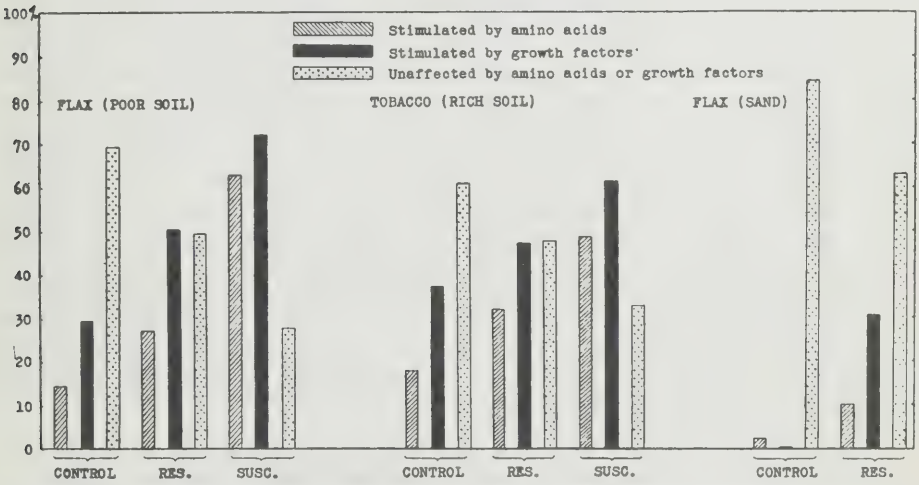

FIG. 1. *Relative incidence of rhizosphere bacteria in relation to certain nutritive requirements.*

Requirements of Bacteria from Tobacco Rhizosphere

The above experiments were repeated with bacteria from the rhizosphere of another crop, grown under field conditions. Two varieties of tobacco, one resistant and the other susceptible to black root rot, were selected for this study. As previously described, 100 isolates from the rhizospheres of resistant and susceptible tobacco varieties, together with 100 controls, were inoculated into each of the four differential media. The results of this com-

parison are also shown in Table I and Fig. 1. The rhizosphere of resistant plants showed an increase of 80% in the relative incidence of bacteria stimulated by amino acid nitrogen, while the susceptible plant rhizosphere showed an increase of 180% compared to the control soil. The number of organisms responding to the medium containing growth factors was increased 29% in the rhizosphere of resistant tobacco, and 64% in the rhizosphere of susceptible tobacco, beyond their incidence in control soil. These results are essentially in agreement with those obtained with flax, which suggests that the same fundamental factors are operative in both cases. As the tobacco soil was relatively rich, however, considerably more thiamin and biotin were already present from decomposing plant residues, thus minimizing somewhat the rhizosphere effect exerted by the plant. The poorer soil in which flax had grown therefore showed the same result in a more accentuated manner.

Relation of Control Soil to Rhizosphere Bacteria

In order to avoid entirely the interfering effect of growth substances already contained in the soil, sand was substituted in another experiment, so that the only source of growth stimulants could be the growing plant. The sand was sterilized in pots, then planted with Bison flax seedlings and inoculated with pure cultures of soil organisms, some requiring accessory growth substances, others requiring amino acids, and others growing well on the simple base medium. The initial total count, which includes approximately equal numbers of each nutritive type, was 12,800,000 per gram of sand. After three weeks in the greenhouse, representative organisms were isolated from the flax rhizosphere, together with controls from corresponding pots in which no plants had grown. The control sand contained 33,000,000 organisms per gram, but only 2% of 100 representative organisms required amino acids for their growth, while no organisms for which growth factors were essential had survived at all. On the other hand, the rhizosphere contained 1,035,000,000 organisms per gram, and, as shown in Fig. 1, approximately one-third of the total numbers was represented by bacteria for which a supply of growth factors is essential.

Thus it appears that unless the more fastidious organisms of the rhizosphere have access to certain organic substances, their development, especially among other forms which are limited by an external supply of these stimulants, is almost impossible. As has been demonstrated above, the numbers of organisms with specific growth factor requirements that occur normally in soils are therefore related in a general way to the organic matter content of the soil in question. The rich tobacco soil supported relatively high numbers, the poorer flax soil fewer numbers, and the infertile sand supported none of the bacteria having more complex nutritive requirements (Fig. 1). Even in sand, however, the predominant types of bacteria immediately adjacent to the plant roots are the more complex forms.

Essential Amino Acids

Organisms for which amino nitrogen was essential were selected for further study, in order to determine which of the ingredients of the amino acid enriched medium was specifically responsible for the observed effects. When each amino acid was tested singly no growth occurred in any case, with the possible exception of cysteine, which produced a barely detectable response. Apparently then, a combination of amino acids was necessary to yield the marked stimulative effects previously observed. On the assumption that cysteine was an essential part of such a combination, this acid was tested with each of the other ingredients of the medium. Results for two typical organisms of this group, shown in Table II, indicate that either alanine or asparagine is effective in combination with cysteine, while aspartic acid and glycine are relatively inert. By varying the concentration of cysteine in the presence of a constant amount of alanine, the optimum effect of the former was observed over the range 0.005 to 0.10 gm. per litre. Higher concentrations of cysteine than 0.01% were distinctly inhibitory. At no concentration could thioglycollic acid replace the amino acid.

TABLE II

COMBINED EFFECT OF CERTAIN AMINO ACIDS ON SELECTED ORGANISMS FROM THE RHIZOSPHERE

Additions to base medium in gm. per l.	Organism	
	XVIII-33	XVIII-21
Asparagine, 0.5	−	−
Aspartic acid, 0.1	−	−
Cysteine, 0.1	±	±
Glycine, 0.1	−	−
Alanine; 0.1	−	−
Ammonium chloride, 0.1	−	−
Cysteine, 0.1 + aspartic acid, 0.1	±	±
Cysteine + asparagine, 0.5	++++	++++
Cysteine + glycine, 0.1	++	+
Cysteine + alanine, 0.1	++++	++++
Cysteine + ammonium chloride, 0.1	+	+
Combination of all above amino acids	++++	++++

An examination of 16 pure amino acids was then made to determine whether any other sources of amino nitrogen, in combination with cysteine, were capable of providing the essential conditions for growth of this group of rhizosphere bacteria. The results of this experiment, presented in Table III, indicated that the only other amino acids able to replace alanine or asparagine were proline or hydroxyproline. the latter possessing somewhat less activity than the others. All exerted full stimulative effect as low as 0.05 gm. per litre, becoming inactive at approximately one-tenth that concentration.

The interesting question concerning whether the plant provides the rhizosphere organisms with one of these essential combinations naturally arises.

TABLE III

AMINO ACID REQUIREMENTS OF BACTERIA FROM THE RHIZOSPHERE

Additions to base medium in gm. per l.	Growth after 5 days at 28° C.		
	XVIII-33	XVIII-21	XVIII-32
Cysteine, 0.1	±	±	±
Cysteine + glycine, 0.1	+	+	++
Cysteine + alanine, 0.1	++++	++++	++++
Cysteine + serine, 0.1	±	±	±
Cysteine + valine, 0.1	±	±	±
Cysteine + leucine, 0.1	±	±	±
Cysteine + phenylalanine, 0.1	±	±	±
Cysteine + tyrosine, 0.1	±	±	±
Cysteine + tryptophane, 0.1	±	±	±
Cysteine + proline, 0.1	++++	++++	++++
Cysteine + hydroxyproline, 0.1	++++	+++	+++
Cysteine + aspartic acid, 0.1	±	±	±
Cysteine + glutamic acid, 0.1	±	±	±
Cysteine + histidine, 0.1	±	±	±
Cysteine + arginine, 0.1	±	±	±
Cysteine + lysine, 0.1	±	±	±
Cysteine + asparagine, 0.1	++++	++++	++++

In Table I it will be noted that 44% of the total numbers of organisms in the rhizosphere of Novelty flax found some such effective combination of amino acids or its equivalent, essential to their growth. The significance of these specific nutritive requirements in the light of possible plant excretions must await further study.

Essential Growth Factors

A determination of the active ingredients in the more complex medium, which contained, in addition to amino acids, a mixture of accessory growth factors, was carried out in a manner similar to the above. Each growth factor, when added to the base medium singly, was without effect. Further experiments showed that the activity of the growth factor mixture was dependent on the presence of cysteine, none of the other amino acids being of importance for this group. Using a base medium containing 0.01% cysteine, each growth substance was tested singly on the bacteria for which growth substances had been found either essential or stimulative, with the results presented in Table IV. This table includes the effect of the accessory growth factors on representative organisms from the rhizospheres of both flax and tobacco. In every case it will be noted that the active ingredient in this medium is either thiamin or biotin or both. β-Alanine, nicotinic acid, and inositol do not appear to be of significance in the nutritive needs of these bacteria. Again the possibility of root excretions suggests itself as an explanation for the relative abundance of thiamin- and biotin-requiring organisms in the rhizosphere compared to soil apart from the plant. Since the rhizosphere

TABLE IV

GROWTH FACTOR REQUIREMENTS OF 25 REPRESENTATIVE RHIZOSPHERE BACTERIA

Organism	Base medium + cysteine, 0.1 gm. per l.					
	Control	Thiamin, 0.1γ per ml.	Biotin, 0.0005γ per ml.	β-Alanine, 0.1γ per ml.	Nicotinic acid, 0.1γ per ml.	i-Inositol, 0.1 mg. per ml.
Flax						
XVI-1	+	+++	++++	++	+	+
XVI-25	−	++++	++++	±	−	−
XVI-43	−	++++	+++	+	−	−
XVI-72	−	+	++++	−	−	−
XVI-82	±	+	++++	+	+	±
XVI-91	−	++++	+++	±	±	−
XVII-5	−	++++	−	−	−	−
XIX-1	−	++++	++++	−	−	−
XVIII-2	−	++	++++	−	−	−
XVI-8	±	±	++++	±	±	±
XVI-13	++	+++	++++	++	++	++
XVI-32	++	+++	++++	++	++	++
XVI-36	+	+++	++++	+	+	+
Tobacco						
C62	−	−	++++	−	−	−
C68	−	−	++++	−	−	−
C89	−	++	++++	−	−	−
C 5	−	−	++++	−	−	−
C19	−	+++	++++	−	−	−
R11	−	−	++++	−	−	−
R82	−	−	++++	−	−	−
S66	−	−	++++	−	−	−
S22	−	++++	++++	−	−	−
S77	−	++	++++	−	−	−
B16	−	++++	−	−	−	−
B 9	−	−	++++	−	−	−

effect is exerted by the plant even in the seedling stage, it appears that the liberation of growth substances into the rhizosphere occurs more probably by excretion than by decomposition. More recent research has yielded direct evidence in support of this view (4).

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS: V. NUTRITIONAL REQUIREMENTS OF THE PREDOMINANT BACTERIAL FLORA[1]

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Though microorganisms concerned with known biochemical processes in the soil have been widely studied, much less attention has been paid to groups of bacteria of which the functions are still largely unknown or but little understood, but which doubtless comprise a large proportion of the indigenous microflora of arable soils. Investigations of the latter groups have been hampered by lack of satisfactory methods for their isolation and of criteria for their classification, both morphological and physiological. Thus in contrast to those organisms concerned with known functions, they require for their isolation as nonselective a medium as possible, in order that "representative" types may develop with the least suppression by other special groups that might be favored by the incorporation in the medium of special energy sources. The procedure is essentially qualitative, though the value of such is greatly enhanced when at the same time quantitative aspects can be taken into account in establishing the relative incidence in any given soil of the various qualitative groups.

For classification of the indigenous soil bacteria on a physiological basis, it is becoming more apparent that the "classical" biochemical tests are inadequate for any rational grouping helpful to an understanding of the activity or significance of these organisms in soil. Furthermore, as Lochhead and Taylor (3, 7) have pointed out, the majority of soil bacteria display a relatively low degree of biochemical activity, as judged by standard laboratory tests, and are regarded as being comparatively unstable physiologically. Hence, other physiological criteria are felt to be more suited to their grouping.

It is self-evident that the equilibrium between various groups of organisms existing in any soil at any given time will depend in large measure upon the availability of nutrients required for the growth of those organisms. Though special antagonisms and the presence of toxic factors may distort this relationship, in the main the relative incidence of a group of microorganisms requiring, for example, a special growth factor will depend upon the presence of that factor in the soil. By classifying organisms according to certain nutritional needs, West and Lochhead (10) suggested a method for measuring the bacterial equilibrium in soil. This method has been applied to advantage by West and Hildebrand (1, 11) in studying the relationship of the soil microflora to strawberry root rot disease and its control. The procedure consisted in observing the growth response of soil bacteria, isolated by nonselective plating methods, in

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three media of varying complexity—basal, amino acid, and growth factor media. The present studies were planned to examine more extensively the nutritional requirements of soil bacteria, with consideration also of those for which the above media are inadequate.

EXPERIMENTAL

The organisms studied were isolated from soils of a 4-year rotation system of oats, clover, timothy, and mangels. One soil (N) had been impoverished by continuous cropping without fertilizer addition; the other (X) had been maintained at good fertility level by regular application of farmyard manure. Samples were taken from each soil in June and in October, 1941.

Cultures were obtained by plating on a nonselective soil extract agar medium according to methods previously described (4, 7). Colonies were systematically picked so tnat all on a plate or a sector were taken, and stab cultures were made into soil extract semisolid (containing 0.02 per cent K_2HPO_4, 0.01 per cent yeast extract, and 0.3 per cent agar) for further study.

Media for nutritional grouping

For a comparative study of the nutritional requirements of the isolates seven media, as follows, were used:

Basal medium (*Medium B*): glucose 1.0 gm.; K_2HPO_4, 1.0 gm.; KNO_3, 0.5 gm.; $MgSO_4$, 0.2 gm.; $CaCl_2$, 0.1 gm.; NaCl, 0.1 gm.; $FeCl_3$, 0.01 gm.; distilled water, 1 liter. Heat to 100°C., filter, and adjust reaction to pH 6.8.

Amino acid medium (*Medium A*): basal medium plus 0.05 gm. per liter each of cysteine, alanine, proline, asparagine, glutamic acid, aspartic acid, arginine, leucine, glycine, and lysine.

Growth factor medium (*Medium G*): basal medium plus cysteine, 0.05 gm.; thiamin, 100 μgm.; biotin, 0.1 μgm; pyridoxin, 200 μgm.; pantothenic acid, 100 μgm.; nicotinic acid, 100 μgm.; riboflavin, 200 μgm.; and inositol, 0.05 gm. per liter.

Amino acid + growth factor medium (*Medium AG*): basal medium plus amino acids as in medium A and growth factors as in medium G.

Yeast extract medium (*Medium Y*): basal medium + yeast extract (Difco), 1.0 gm. per liter.

Soil extract medium (*Medium S*): 750 ml. basal medium plus 250 ml. soil extract, prepared by autoclaving 1 kgm. soil with 1 liter water for 30 minutes, filtering after adding a little $CaSO_4$, and making filtrate up to 1 liter.

Yeast + soil extract medium (*Medium YS*): soil extract medium plus 1.0 gm. yeast extract per liter.

All media were tubed in 5-ml. amounts. Transfers of each organism were made by loop inoculation from soil extract semisolid. Cultures were incubated at 26°–28° C. for 5 days and the growth responses of each isolate in the seven media recorded by assigning a turbidity value of 4 to the tube showing heaviest growth and rating the others by comparison. To avoid assigning too great importance to small variations, a difference of not less than 2 points was considered significant. Readings of 1 and 2 were regarded as showing submaximal growth.

On the basis of their growth in the seven media, the organisms could be divided into the following groups:

I. *Bacteria with simple requirements.* These bacteria show good growth in medium B and are therefore capable of synthesizing their requirements for maximum growth from ingredients of the glucose-nitrate-salts medium. Growth is not significantly better in any of the other media.

II. *Bacteria requiring one or more amino acids.* These organisms grow well in medium A but show either no growth or submaximal growth in medium B. Those which grow equally well in medium A and G obviously require only cysteine since this is common to both. The majority of those showing maximum development in A, however, do not grow well in G, indicating a need for one or more of the other amino acids present in the medium.

III. *Bacteria which require growth factors.* These bacteria produce maximum growth in medium G but none or submaximal growth in B or A. Previous work (9) has shown that response to growth factors occurs best in the presence of cysteine, and that the more important growth factors are thiamin and biotin.

IV. *Bacteria requiring amino acids plus growth factors.* The bacteria in this group show maximum development in medium AG, but show no growth or only submaximal growth in B, A, or G. Evidently, in addition to one or more growth factors, these organisms require one or more amino acids of medium A apart from cysteine.

V. *Bacteria requiring unidentified substances in yeast extract.* These organisms show maximum growth in medium Y, but fail to grow, or develop only slightly, in B, A, G, or AG. In the soil extract medium (S) growth in the great majority of cases is absent or meager, though a few members of the group show growth equal to that in Y.

VI. *Bacteria requiring unidentified substances in soil extract.* Organisms of this group are capable of maximum development in medium S, but show none or submaximal growth in B, A, G, AG, or Y.

VII. *Bacteria requiring unidentified substances in both yeast extract and soil extract.* These bacteria are capable of maximum development only in medium YS. In all of the media B, A, G, AG, Y, and S, growth is either absent or submaximal.

It is obvious that further classification into subgroups could be made on the basis of growth response in the media selected. Thus distinction could be made between organisms showing no growth and those capable of submaximal growth in the basal medium or in other media. For the main purpose of this report classification is based on the seven main groups.

Incidence of different nutritional groups in soil

The relative incidence of the various nutritional groups of bacteria in the soils studied is shown in table 1. In the fertile soil, organisms capable of developing in the basal medium, or indeed in the "synthetic" media of known composition (groups I to IV), were less abundant than in the poor soil (39.4 per cent as compared with 52.8 per cent). On the other hand forms for which the more complex ingredients of yeast and soil extract are necessary (groups V to VII) were relatively more numerous. Despite such differences, doubtless related to the higher content of organic matter in the better soil, the incidence of the different groups

does not appear to reflect markedly the pronounced difference in crop-producing capacity. The classification of bacteria on the basis of nutritional requirements, however, indicates certain differences between soils not brought out by classification based on morphology (7).

Table 1 also gives the bacterial groups as determined at different seasons. In October, organisms requiring amino acids and growth factors for maximum growth were less abundant than in June, a result which appears explicable in view of the effect of an actively growing crop. In other respects little differences between the two sampling dates were noted.

TABLE 1

Nutritional groups of soil bacteria in relation to fertility and season

	SOIL N (INFERTILE)		SOIL X (FERTILE)		JUNE SAMPLING		OCTOBER SAMPLING	
Number of cultures on soil extract—yeast semi-solid	171		165		175		161	
	no.	*per cent*	*no.*	*per cent*	*no.*	*per cent*	*no.*	*per cent*
Nutritional group								
I. Good growth in basal medium	25	14.6	13	7.9	18	10.3	20	12.4
II. Require amino acids	14	8.4	20	12.1	18	10.3	16	9.9
III. Require growth factors	22	12.8	20	12.1	18	10.3	24	14.9
IV. Require amino acids and growth factors	29	17.0	12	7.3	29	16.6	12	7.5
V. Require yeast extract	16	9.4	37	22.4	24	13.7	29	18.1
VI. Require soil extract	12	7.0	5	3.0	11	6.3	6	3.7
VII. Require yeast and soil extract	50	29.2	53	32.1	51	29.1	52	32.3
No growth in test media	3	1.8	5	3.0	6	3.4	2	1.3

Relation of nutritional groups to morphological types

To note any relationship between the nutritional requirements of the organisms and their morphological grouping, all isolates were subcultured on a variety of media, and microscopic observations were made to permit of their classification into broad groups. For this purpose the general procedure of previous work (4) was followed. Special attention was given to the detection of pleomorphic forms related to the group of corynebacteria and which in many soils comprise a numerically important group.

As shown in table 2, the various nutritional groups of bacteria are not restricted to any definite morphological type of organism, but include for the most part representatives of various forms. Some of the less abundant morphological types, however, are restricted to certain groups, and certain trends in the distribution of the more abundant types are noted. Organisms with simpler requirements (groups I and II) consist to a rather larger extent of sporeforming rods and Gram-negative nonsporing rods. In the case of organisms requiring the more complex ingredients of soil extract (groups VI and VII), pleomorphic

forms comprise a greater percentage of the total. There is, however, no clear-cut correlation of morphology with nutritional needs.

Relation to certain biochemical properties

Table 2 also shows the relationship of the different nutritional groups to certain biochemical properties. To provide a medium suited to the growth of all organisms, a base of soil extract with 0.02 per cent K_2HPO_4 and 0.01 per cent yeast extract was used. The test media contained respectively 0.1 per cent KNO_3, 1 per cent dextrose, and 10 per cent gelatin, the first two being semisolid (0.3 per cent agar).

TABLE 2

Nutritional requirements of soil bacteria in relation to morphological type and some biochemical properties

	NUTRITIONAL GROUP						
	I	II	III	IV	V	VI	VII
Total number of cultures	38	34	42	41	53	17	103
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Morphological group							
Cocci, Gram-positive	5.3	3.0	4.8	9.8	15.1	11.8	11.6
Cocci, Gram-negative	5.3	0.0	14.3	4.9	3.8	0.0	0.0
Rods, sporeforming	5.3	17.6	0.0	7.3	3.8	0.0	1.0
Rods, nonsporing, Gram-positive	10.5	11.8	19.0	29.3	13.2	11.8	8.7
Rods, nonsporing, Gram-negative	50.0	52.9	40.5	17.1	41.5	23.5	29.1
Pleomorphic, Gram-positive	23.7	11.8	21.4	31.7	17.0	41.2	46.6
Pleomorphic, Gram-negative	0.0	3.0	0.0	0.0	5.9	11.8	2.0
Physiological group							
Nitrate reduction	42.2	53.1	52.5	61.1	56.3	64.7	64.0
Gelatin liquefaction	67.6	80.7	72.5	66.0	52.2	41.2	57.8
Dextrose 1 per cent—acid	48.6	31.3	52.5	61.1	36.8	53.3	33.6
Dextrose 1 per cent—alkaline	46.0	31.3	7.5	5.6	14.3	0.0	44.5
Inhibited by 1 per cent dextrose	0.0	6.2	2.5	0.0	8.2	6.0	16.4

Ability to reduce nitrates, liquefy gelatin, or produce acid in dextrose does not appear to be associated with any group or groups. Considerable variation was noted in ability to produce an alkaline reaction in the dextrose medium. The effect of dextrose in suppressing growth of many soil bacteria, pointed out previously (7), was again noted. It is seen that the organisms inhibited by the presence of the sugar are mainly those with the most complex nutritional requirements, especially group VII.

ORGANISMS REQUIRING SOIL EXTRACT

The bacteria of groups VI and VII comprised those for which soil extract, with or without yeast extract, was required for maximum growth. Of the 120 cultures in these groups, 57 showed submaximal development in yeast extract

(medium Y). The remaining 63 cultures, comprising 19 per cent of the total isolates from soil, were unable to develop in medium Y (or in media B, A, G, AG or in one containing peptone with the addition of the growth factors) and were given special consideration. All grew well in medium YS, and of these, 8 showed equally good growth in soil extract without yeast (medium S), 42 gave submaximal growth, and 13 no growth.

The morphological distribution of the organisms of this group, indicated below, showed a higher proportion of pleomorphic bacteria and of Gram-positive cocci than any of the other nutritional groups:

	per cent
Cocci, Gram-positive	15.9
Cocci, Gram-negative	1.6
Rods, sporeforming	0.0
Rods, Gram-positive	3.2
Rods, Gram-negative	20.7
Pleomorphic, Gram-positive	57.3
Pleomorphic, Gram-negative	1.6

Effect of treatment on soil extract

Since all organisms grew well in soil extract with yeast extract (medium YS) and showed no growth with yeast extract alone (medium Y-), comparisons were made with all organisms in these media and in various media containing yeast extract to which were added respectively preparations of soil extract subjected to different treatments. Such addenda included soil extract ash; acetone, alcohol, and ether extracts; the filtrate after treatment with Norit; the ammoniacal alcohol eluate of the Norit adsorbate; and a combination of charcoal filtrate and eluate. In all cases the addenda were made to correspond with the volume of soil extract present in the control medium YS.

The effects of the various treatments are summarized in table 3. Soil extract ash was able to provide substances required for maximum growth for only 1 organism, and to permit of submaximal growth, in most cases very slight, of 8 cultures. Of the various solvents used for extraction, acetone was the most effective. The acetone extract permitted growth of all but 11 organisms, 26 showing maximum development. Treatment of soil extract with alcohol was much less satisfactory. The ether extraction removed from soil extract substances suited to the growth of but 1 of the 63 cultures. Treatment with charcoal removed entirely the growth-promoting effect of soil extract for 49 of the cultures and partially for 11 organisms. Only 3 of the 63 cultures were capable of maximum growth with the charcoal filtrate.

Elution of the charcoal with alcohol yielded an eluate which permitted maximum or submaximal growth of 44 of the organisms. The results indicate that at least partial recovery of the adsorbed substances necessary for growth of the majority of the bacteria of this group could be made. A further test, in which the charcoal filtrate and eluate were both present, gave a still higher proportion

of the organisms showing growth. The results suggest either a multiple nature of the factors required for some organisms, or more probably, incomplete adsorption and elution respectively under the experimental conditions.

TABLE 3

Effect of various treatments on growth-promoting properties of soil extract

ADDENDA TO YEAST EXTRACT (MEDIUM Y)	NUMBER OF ORGANISMS		
	No growth	Submaximal growth	Maximum growth
Controls			
Nil	63	0	0
Soil extract, untreated	0	0	63
Soil extract fractions			
Ash	54	8	1
Acetone extract	11	26	26
Alcohol extract	29	27	7
Ether extract	62	0	1
Charcoal filtrate	49	11	3
Charcoal eluate	19	36	8
Charcoal filtrate + eluate	9	38	16

Extracts from soils of different fertility

To note the effectiveness of extracts prepared from soils of different productivity, comparisons were made between the poor soil (N) and the fertile soil (X), the average relative crop-producing powers of which over a 5-year period were as follows:

	Timothy	*Mangels*	*Oats*	*Clover*
Soil N	64	9.5	72	42
Soil X	100	100	100	100

Tests were made with 15 organisms in media prepared with extracts of the two soils incorporated with medium Y. The results, given in table 4, show the pronounced difference in growth-promoting ability of the extracts from the two soils for most of the organisms tested. The good growth of certain cultures with the extract of the poor soil indicates the varied nature of the requirements of different organisms and that more than one growth-promoting substance may be present in soil extract.

Growth-promoting effect of group I organisms

Table 4 also includes results of a test to note whether the nutrilite effect of soil extract could be replaced by a filtrate from cultures of bacteria of group I, capable of synthesizing their growth requirements from the ingredients of the basal medium. Five cultures of group I organisms selected at random were grown in medium B. When maximum growth was reached the cultures were combined

and passed through a Seitz filter. To 3 parts of medium Y, 1 part of filtrate was added. Five of the test organisms grew well and two others responded slightly to the addition of the filtrate, indicating that for some of the more fastidious soil bacteria the growth-promoting effect of soil extract could be supplied by metabolic products of other organisms having simpler requirements. This is of interest in view of the ability of certain soil microorganisms to produce auxins (5).

The filtrates used were ineffective for other test organisms, indicating distinctly different growth requirements and further suggesting the presence of more than one growth-promoting factor in soil extract. Since the number of

TABLE 4

Growth-promoting effect of extracts from soils of different fertility and of filtrates of group I organisms

CULTURE NUMBER	RELATIVE GROWTH* IN MEDIUM Y PLUS ADDENDA			
	Nil	Extract from poor soil (N)	Extract from fertile soil (X)	Filtrate from culture of bacteria of group I
1N13	0	2	4	0
1N18	0	0	4	4
1N51	0	4	4	3
1N69	0	0	4	0
1X17	0	0	4	0
1X21	0	0	4	0
1X55	0	0	4	0
1X67	0	4	4	0
2N2	0	0	4	0
2N7	0	2	4	1
2N11	0	0	4	3
2N18	0	0	4	0
2N20	0	0	4	1
2N40	0	0	4	4
2N58	0	0	3	4

* A turbidity value of 4 indicates heavy growth; lower values indicate correspondingly less growth.

group I organisms used was small, it is by no means improbable that other members of this group would be able to furnish substances suited to other test organisms unable to grow with the filtrates provided. The results emphasize the interdependence of many members of the soil microflora.

DISCUSSION

The classification of soil bacteria on the basis of nutritional needs reveals the presence of groups, varying in requirements from simple to very complex, the relative incidence of which may be fairly constant in soils of given type. Any broad system of nutritional grouping, however, may give a false impression of uniformity in type of the members of a given group. Previous work on morphological and general biochemical classification (7) brought out the variability

inherent in apparently closely related types. The present nutritional studies emphasize the divergencies possible between members of the same group. The nutritional basis of grouping, however, serves as a better aid to any understanding of the function of the organisms in soil.

TABLE 5

Nutritional requirements as indicated by growth in various media, of organisms of group IV (amino acids and growth factors)*

CULTURE NUMBER	CONTROL (MEDIUM AG)	AMINO ACIDS PLUS SINGLE GROWTH FACTORS							GROWTH FACTORS PLUS SINGLE AMINO ACIDS								
		Thiamin	Biotin	Pyridoxin	Pantothenic acid	Nicotinic acid	Riboflavin	Inositol	Alanine	Proline	Asparagine	Glutamic acid	Aspartic acid	Arginine	Leucine	Glycine	Lysine
1N1	4	2	2	0	0	2	0	0	0	3	0	0	0	3	1	0	0
7	4	0	1	1	0	0	2	0	0	0	0	0	0	1	1	0	0
8	4	3	4	1	2	1	2	2	2	2	4	3	4	4	2	3	2
9	4	4	4	4	1	1	2	1	4	1	1	2	1	1	1	1	1
14	4	1	2	0	0	0	0	0	0	0	1	0	0	0	4	1	1
15	4	1	1	0	0	0	1	0	0	1	0	0	0	2	0	1	0
25	4	1	tr	0	0	0	0	0	0	0	0	0	0	0	4	4	0
31	4	2	3	1	1	tr	1	tr	2	2	4	4	3	4	2	1	2
36	4	3	2	0	0	0	1	0	0	0	1	0	0	0	1	0	0
38	4	4	2	1	0	0	0	0	3	3	4	3	2	4	4	1	1
39	4	4	4	2	1	1	1	2	4	4	4	4	3	4	3	4	3
46	4	1	2	0	0	0	0	1	4	4	4	4	4	4	4	4	2
47	4	2	2	1	1	1	0	1	0	3	3	0	1	0	4	0	0
50	4	4	4	1	1	1	2	1	3	1	1	2	1	1	1	1	1
54	4	tr	4	0	0	0	0	0	0	4	3	0	4	4	0	0	4
63	4	4	4	2	1	0	0	0	1	1	1	1	1	2	2	1	1
71	4	4	1	0	0	0	0	0	0	0	0	0	0	1	4	0	0
1X10	4	0	4	0	0	0	0	1	0	0	0	0	0	2	3	0	4
11	4	1	2	0	0	tr	0	0	0	4	3	0	0	4	0	0	3
33	4	3	3	4	3	4	3	3	0	1	0	0	0	0	2	1	0
35	4	1	3	2	0	0	0	0	1	0	0	0	0	0	1	0	0
56	4	4	tr	tr	0	0	0	0	0	0	1	1	1	0	0	0	0
61	4	4	3	0	0	0	2	2	2	4	4	0	0	4	4	4	4
63	4	3	3	2	1	1	1	1	0	2	4	3	4	3	3	0	1
68	4	3	2	0	1	1	1	1	1	0	1	0	0	0	1	4	1
72	4	4	3	1	1	0	0	1	4	3	4	4	1	1	2	3	1

* A turbidity value of 4 indicates heavy growth; lower values indicate correspondingly less growth.

Illustrative of the differences in specific nutritional requirements between organisms of the same group are results from a more detailed study of cultures of group IV, all requiring amino acids plus growth factors for maximum growth. Each culture was inoculated into 17 different media, including medium AG as control and two series of media, one with all amino acids plus single growth factors, and one with all growth factors plus single amino acids. From table 5 it is

seen that within the group there are wide differences in individual nutritional requirements. The results suggest that the indigenous soil bacteria may exercise highly specialized functions, such as the utilization of specific chemical compounds formed during the process of decomposition of organic matter. The variability brought out by more detailed study of related organisms further emphasizes the difficulty in classification of soil bacteria.

There is comparatively little exact knowledge of the occurrence of bacterial growth factors in soil. The presence of vitamins of the B group, most of which are important for the nutrition of many bacteria, is probable in soils receiving organic fertilizer. Sanborn (6) has shown that during the decomposition of plant material in soil, growth accessory factors for cellulose-decomposing bacteria may be elaborated. Lilly and Leonian (2) have demonstrated the presence of thiamin, and the occurrence of biotin in plant and animal tissue and in manure may well account for its presence in well-fertilized soils. Furthermore West (8) has shown that measurable quantities of thiamin and biotin may be excreted by the roots of seedlings.

Results from our studies with organisms requiring soil extract suggest the presence of other, probably unknown, microbial growth factors in soil. This group showed no growth with the nutrilite supplement, which included thiamin, biotin, riboflavin, pyridoxin, pantothenic acid, nicotinic acid, and inositol, suggesting that the growth-promoting properties of soil extract for this group are to be ascribed to factors other than those named. Differences in response of certain organisms to extracts from different soils as well as to the nutrilite effect of filtrates of other soil organisms appear to indicate the presence of more than one such growth-promoting factor in soil.

SUMMARY

Based on a determination of growth requirements, a classification was made of soil bacteria isolated by nonselective plating methods. Seven main nutritional groups were recognized, ranging from organisms capable of maximum development in a simple basal medium to types unable to develop with supplements of amino acids, growth factors, or yeast extract, but which require soil extract for growth. The more fertile soil of two studied showed a higher proportion of types with more complex growth requirements than the poorer soil.

More detailed study of the requirements of individual cultures of one group (requiring amino acids and growth factors) showed wide variations between related forms. The results suggest that many soil bacteria exercise functions of a highly specialized nature.

Correlation of nutritional requirements with morphological type did not reveal any clear-cut relationship, though certain trends were noted. Organisms with simpler requirements consisted to a larger extent of sporeforming rods and Gram-negative nonsporing rods. Pleomorphic forms related to corynebacteria comprised a greater proportion of organisms with more complex nutritional needs.

Bacteria requiring soil extract for growth comprised 19 per cent of the isolates

from soil. For the great majority of these forms, the growth-promoting properties of soil extract were dependent upon a factor or factors not concerned with the ash constituents, but present in the acetone extract and capable of adsorption by charcoal and of recovery by elution. Since the organisms showed no growth with a combination of seven known growth factors, it is suggested that the growth-promoting properties of soil extract for this group of bacteria are to be ascribed to factors other than these. Soils may vary greatly in the effectiveness of extracts prepared from them for certain organisms.

For certain organisms requiring soil extract, the nutrilite effect of the latter could be replaced by a filtrate from cultures of other soil bacteria capable of maximum development in simple basal medium. For other organisms, similar filtrates were ineffective, indicating different growth requirements and suggesting that soil extract contains more than one growth-promoting factor.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS: VI. INFLUENCE OF SEASON AND TREATMENT ON INCIDENCE OF NUTRITIONAL GROUPS OF BACTERIA[1]

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Recent publications from this laboratory (4, 6) have drawn attention to the nutritional requirements of soil bacteria developing on a nonselective medium (soil extract agar). On the basis of these studies a classification was proposed in which these bacteria were grouped according to their nutritional needs. In addition, it was emphasized that the organisms representing these groups were in equilibrium with one another, an equilibrium which seasonal change and soil treatment might disturb. Some results of studies on the influence of these two factors on the incidence of certain bacterial nutritional groups in soil are reported herein.

FIELD PLOT STUDIES

Soil samples were taken periodically (usually at monthly intervals) at a depth of 2 to 6 inches from two plots of different manurial treatment: N—no fertilizer; X—farmyard manure. The plating medium and analytical procedures employed are described elsewhere (3, 5, 6).

The results obtained over a period of 21 months are presented in figure 1 and represent the bacterial response to the combined effects of season, crop, and soil treatment. Organisms with very simple food requirements (group A, those growing well in mineral-glucose medium) were very prominent in the fall and winter of 1940, declined in the summer of 1941, and tended to rise again (though not to the same heights as in 1940) in the following fall and winter, whereas bacteria which require yeast extract (group E) varied more or less conversely. Bacteria requiring known amino acids (groups B and C) or growth factors (group D) showed some fluctuation but none which could be correlated with change of season. On the other hand, organisms growing only in soil extract semisolid agar (group F)[3] fluctuated fairly regularly with the season, being low in the late fall and winter and high in the early spring and summer.

It appears that group F is more sensitive to seasonal changes than the other groups but is not appreciably affected by soil treatment or the nature of the crop. Groups B, C, and D do not seem to be significantly affected by any of

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[2] The authors wish to express their thanks to A. G. Lochhead, Dominion agricultural bacteriologist, for suggesting this problem and for his sustained interest throughout the course of this work.

[3] This group of bacteria was originally included in group E by West and Lochhead (6) and is roughly comparable to groups VI and VII of Lochhead and Chase (4).

the factors involved. The variations of groups A and E, however, point to the influence of at least two factors—season and crop. Timothy, grown in 1940, may have stimulated bacteria of group A or depressed those of group E,

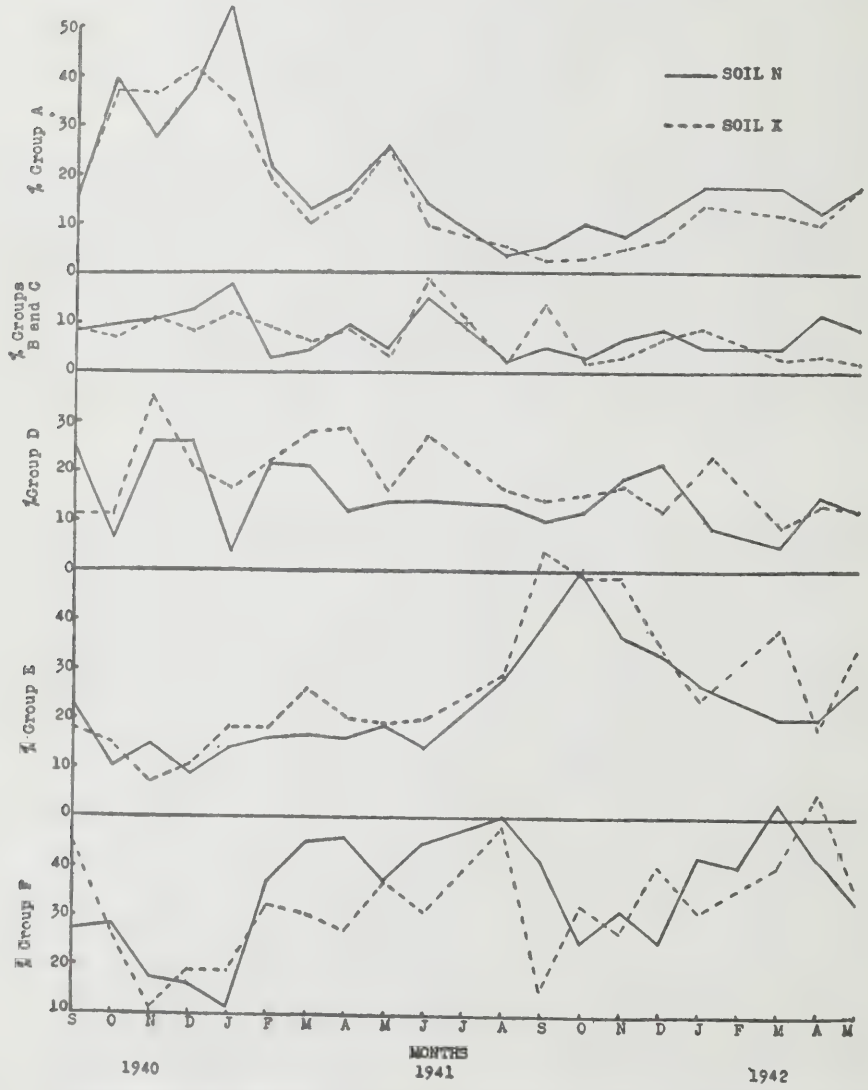

FIG. 1. INFLUENCE OF SEASON ON INCIDENCE OF CERTAIN BACTERIAL NUTRITIONAL GROUPS IN SOIL

whereas mangels, planted in 1941, may have induced an opposite response, resulting in an increase of group E or a decrease of group A. Taylor and Lochhead (5), working with the same soils, obtained "increased counts of Gram-positive and Gram-variable short rods and *Bacterium globiforme*" after mangels

as compared with numbers following timothy. Thus it is quite possible that the nature of the crop exerts an influence as profound as, if not more profound than, the effect of season on specific groups of soil microorganisms.

Further analysis of figure 1 shows that though soil treatment did not influence significantly the seasonal fluctuations of the various groups, it did govern their abundance. Soil X—receiving manure—supported, more or less consistently, larger numbers of bacteria requiring specific growth factors or yeast extract (groups D and E respectively), and soil N—untreated—, larger numbers of bacteria with very simple nutritional needs (group A). Lochhead and Chase

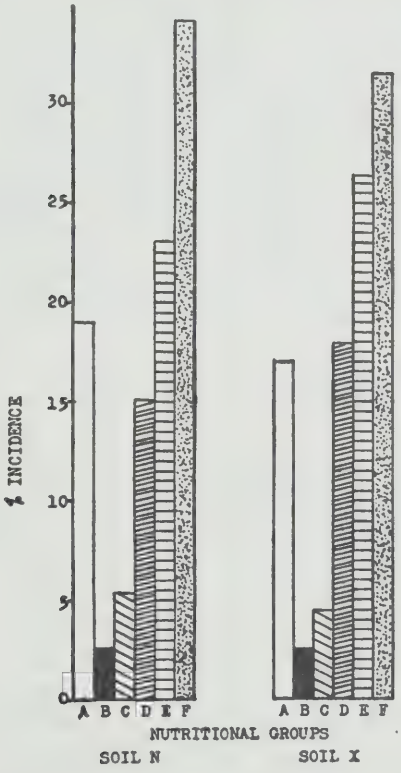

FIG. 2. RELATIVE INCIDENCE OF BACTERIAL NUTRITIONAL GROUPS IN SOIL

(4) working with the same plots also noted that soil X was more favorable to group E and less favorable to group A than soil N. The results with group D are not comparable, as the constituents of the growth-factor medium used were not identical in the two experiments.

Despite the differences noted, the average relative incidence of the nutritional groups over the entire sampling period was found to be remarkably similar in both soils. As may be seen in figure 2, organisms of group F were the most numerous, closely followed by those of group E; groups A and D were next in abundance, and B and C last. This suggests that the bacterial equilibrium

in these two soils has not been changed appreciably by treatment. Taylor and Lochhead (5) also point out that "in a soil of a given type the bacterial flora may be fairly resistant to change even though the productivity may vary as much as ten-fold."

POT EXPERIMENTS

According to the work of West and Hildebrand (1, 7), treatment of soil with red clover, manure, soybeans, carbohydrates, and acetic acid caused an appreciable shift in the bacterial equilibrium of the soil as indicated by their measure of this equilibrium—the bacterial balance index (6). For example, the addition of soybeans, carbohydrates, and acetic acid induced a high bacterial balance index, which means that a relative increase of organisms requiring known amino acids and growth factors and a decrease of bacteria with very simple nutritional needs occurred. This shift in equilibrium was found to be associated with a decrease in severity of strawberry root rot in their soil. The experiments reported below were designed to yield further information concerning the effect of different soil amendments on the equilibrium between the bacterial nutritional groups in the soil, particularly with a view of inducing a high bacterial balance index.

Strawberry root rot soil from St. Catharines, Ontario, received the following treatments:

SERIES	TREATMENT	SERIES	TREATMENT
Control soil	None	E	1% dextrose
A	3% green strawberry plants	F	5% dextrose
B	5% green soybean plants	G	5% soluble starch
C	5% green red clover plants	H	5% molasses
D	5% ground filter paper	I	Acetic acid to pH 4.5

These treatments were applied to the soil at 60 per cent of its moisture-holding capacity, thoroughly mixed, and the mixtures placed in $\frac{1}{4}$-gallon crocks in duplicate; the crocks were covered, weighed, and maintained at room temperature (20–25°C.). Water was added periodically to compensate for loss by evaporation. Samples for analysis were removed at suitable intervals; the analytical procedures employed were the same as in the field plot studies.

Influence of treatment on the relative incidence of bacterial nutritional groups

Although samples were taken at three periods—40, 120, and 360 days—the data for only the first and last are summarized in table 1, the results of the second being intermediate. After 40 days group A was lower in soils treated with molasses, starch, 5 per cent dextrose, and especially cellulose than in the control soil. With time, this group declined in abundance in all soils except the molasses-treated, which remained virtually unchanged, and the cellulose-treated, in which the group became particularly prominent. In general, after 360 days, the percentage incidence of these bacteria was somewhat greater in the treated soils than in the control.

Bacteria requiring amino acids were markedly stimulated by cellulose and soluble starch. Soybeans, dextrose, starch, and acetic acid were also favorable, a fact which explains, in part, the higher bacterial balance index in these soils than in the control. After 360 days the effect was still noticeable in soils treated with soybeans and dextrose. Red clover also induced an increase of these organisms at this time.

Organisms with specific growth factor requirements were only moderately stimulated by the treatments. At the end of the experimental period, however, certain of the amendments such as soybeans and cellulose seemed actually to depress the group.

TABLE 1

Influence of soil amendments on the incidence of certain bacterial nutritional groups

SOIL TREATMENT	GROUP A, GROWING WELL IN MINERAL-GLUCOSE MEDIUM		GROUPS B & C, REQUIRING AMINO ACIDS		GROUP D, REQUIRING KNOWN GROWTH FACTORS		GROUP E, REQUIRING YEAST EXTRACT		GROUP F, GROWING IN SOIL EXTRACT SEMISOLID AGAR ONLY		BACTERIAL BALANCE INDEX	
	40 days	360 days	40 days	360 days	40 days	360 days	40 days	360 days	40 days	360 days	40 days	360 days
	%	%	%	%	%	%	%	%	%	%		
Control soil	37	7	8	8	18	26	12	46	25	13	−4	+29
3% Strawberry tissue	50	17	8	8	27	18	9	26	6	31	−17	+18
5% Soybean tissue	37	28	15	14	31	8	8	42	9	8	+17	+11
5% Red clover tissue	38	6	9	19	26	16	18	40	9	19	−2	+32
5% Cellulose	6	63	48	9	20	4	12	20	14	4	+51	−56
1% Dextrose	41	26	4	16	22	11	4	29	29	18	−25	+15
5% Dextrose	22	14	16	15	25	26	37	31	5	14	+7	+29
5% Soluble starch	16	13	28	7	21	23	30	44	5	13	+26	+10
Molasses	12	15	17	3	36	18	25	40	10	24	+23	+23
Acetic acid to pH 4.5	29	9	17	5	32	14	20	42	2	29	+22	+12

After 40 days' incubation, group E was higher in soils with red clover, 5 per cent dextrose, starch, molasses, and acetic acid than in the control. There was a tendency for this group to increase with time, until at 360 days 40 per cent of the isolates belonged to it in six of the soils including the control. Certain treatments such as cellulose again appeared to repress these bacteria.

Group F was depressed by most of the treatments at 40 days. After 360 days, however, it was more abundant than in the control in soils receiving strawberry tissue, molasses, and acetic acid. Once more, cellulose seemed to exert an inhibitory effect.

The foregoing analysis of the data in table 1 shows how extensively certain treatments can modify the incidence of some bacterial nutritional groups in soil and how the latter may vary even in untreated soil, a phenomenon which was recorded previously (2). On the basis of these results, it is difficult to

generalize as to the effect of broad types of substances as proteinaceous materials or carbohydrates on the groups of bacteria studied. For example, the two legumes, both comparatively rich in nitrogen, produce different initial and residual effects, as do the various carbohydrates used. The quantity of the treatment may also induce a different response (compare 1 and 5 per cent dextrose).

Bacterial balance index

The influence of various amendments on the bacterial nutritional groups in soil is brought out very clearly by the bacterial balance index of West and Lochhead (6). The results after 40 days (table 1), on the whole, corroborate those of West and Hildebrand (1, 7) who found that soybeans, dextrose, and acetic acid increased the "B.B.I." of root-rot soil, whereas red clover was comparatively ineffective. The data obtained in the present work indicate that other carbohydrates (starch, cellulose, and molasses) also increased the index. On the other hand, strawberry tissue lowered it. Since this material is incorporated in soil under ordinary farming practices, it may be one of the factors responsible for the shift of the bacterial equilibrium in soil to a point where it becomes unfavorable to the plant, and a root-rot condition develops (1, 7). Again, the quantity of material used appears to exert a very important effect: 1 per cent dextrose actually depressed the index to −25, whereas 5 per cent increased it to +7. Larger quantities may well raise the index farther (7). Residual effects of treatments must also be considered, as is borne out by the results with cellulose: after 40 days this material gave an index of +51, but after 360 days the equilibrium had shifted to an extremely low level of −56. The tendency for the "B.B.I." of the control soil to increase with time was observed previously (2) and may reflect a natural tendency of normal soil which has been removed from the field, dried, remoistened, and incubated at room temperature to reach an equilibrium with its new environment.

DISCUSSION

In a given soil environment, the components of the microbial population are in equilibrium with one another. Any change in this environment produced by season, growing crop, or soil treatment may shift this balance to a new one which is a reflection of the biological activity of the new factor. This applies to the interrelationships not only among broad groups of microorganisms such as protozoa, fungi, or bacteria but also among the constituent members of these groups. To what extent the new equilibrium will persist depends on the quantitative and qualitative intensity of the new environmental factor and the length of time over which it operates, because it is obvious that the inherent soil properties which are the result of thousands of years of adaptation will not be altered appreciably by a superficial treatment applied over a comparatively short period of time. It has been pointed out earlier (fig. 2) that despite the different productivity of soils N and X (as a result of treatment) the relative incidence of the bacterial nutritional groups studied is very similar in both soils, an observation

which Taylor and Lochhead (5) have also made. Continuous planting of one crop may be expected eventually to stabilize the microbial balance of the soil and result in a microflora which is characteristic of that crop. Annual change of crop, as in rotations, however, will merely cause temporary shifts of the equilibrium (groups A and E in figure 1). Similarly, readily decomposable materials (dextrose) stimulate a temporary change in the equilibrium, which eventually reverts to its original state as a result of the powerful buffering capacity of the soil, whereas more slowly decomposable substances (cellulose) induce a shift of the bacterial balance which is not only more profound but also more persistent.

These considerations are especially important if it is desired to modify the soil population for a specific purpose such as the elimination of a disease factor from soil or the stimulation of a microflora favorable to a particular crop.

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QUALITATIVE STUDIES OF SOIL MICRO-ORGANISMS

VII. THE 'RHIZOSPHERE EFFECT' IN RELATION TO THE AMINO ACID NUTRITION OF BACTERIA[1]

By A. G. Lochhead[2] and R. H. Thexton[3]

Abstract

Comparative studies of the relative incidence of bacteria of different nutritional requirements in soil indicate that one of the most characteristic rhizosphere effects is the preferential stimulation of bacteria requiring amino acids for maximum growth. Organisms for which amino acids are either essential or stimulative were proportionately increased in the rhizosphere. No similar effect was noted with respect to bacteria responding to growth factors.

The findings suggest, by indirect evidence, that the effect is to be ascribed to the excretion of amino acids by the growing plant. However, though this may be the chief factor, the preferential stimulation of the amino acid group of bacteria may be related to associative and antibiotic effects exerted by other bacteria, stimulated in the rhizosphere, observed to have different degrees of compatibility towards those responding respectively to amino acids and growth factors.

Introduction

It is now well established that the growing plant may exert a marked effect, both quantitative and qualitative, on the soil micro-organisms within the zone of influence of the root system (rhizosphere). Soil in the vicinity of the roots supports usually noticeably higher numbers of organisms than soil more distant from the plant, the effect varying with such factors as the kind of crop, the age of the plants, soil treatment, and moisture conditions (1, 3, 5, 11, 12, 14, 15). The rhizosphere of diseased plants may contain greater concentrations of micro-organisms than that of healthy plants (4, 13), while there is evidence that varieties susceptible to certain soil-borne diseases, even though healthy, may show higher numbers of organisms in the rhizosphere than corresponding resistant varieties (15). The 'rhizosphere effect' may be shown, not only in total numbers, but in a preferential stimulation of certain physiological groups of micro-organisms, as judged by quantitative determinations with selective media (2, 3, 5, 7, 8, 11, 12).

Earlier papers in this series (9, 17) have dealt with the influence of plant growth on the characteristics of soil bacteria studied by a quanti-qualitative procedure based on single culture isolations from a non-selective medium, chosen to permit the most nearly representative growth of the indigenous microflora. On the basis of morphology and taxonomic relationship, as well as physiological characters, Lochhead (9) found marked differences in the predominant bacteria in the rhizosphere as compared with soil distant from plant roots, similar trends being noted with all crops studied—clover, oats,

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mangels, corn, tobacco, and flax. In a study of the nutritional requirements of bacteria, West and Lochhead (17) showed that the relative incidence of bacteria for which amino acids and growth factors were essential or stimulative was higher in the rhizosphere of flax and tobacco plants than in corresponding control soils. Using a similar procedure, Katznelson and Richardson (6) found a higher proportion of bacteria stimulated by amino acids in the rhizosphere of tomato plants than in the control soil.

In the nutritional studies referred to, classification of the bacteria was made by observing relative growth in three media, consisting of a basal medium without addenda, and with the addition of amino acids or known growth factors. While these media permit a certain grouping of the organisms, a more complete differentiation, based on nutritional requirements, was provided by the procedure of Lochhead and Chase (10), which involved a determination of growth response in seven cultural media ranging from a simple basal medium to those containing amino acids, growth factors, amino acids with growth factors, or the unidentified substances present in yeast or soil extract or both. This report deals with the rhizosphere effect on the predominant soil bacteria classified on this basis.

Experimental

Bacteria were isolated from control soil and from the rhizosphere of mangels from a four-year rotation system with oats, clover, and timothy. The soil had been maintained at good fertility level by regular application of farmyard manure. For the rhizosphere sample, soil adhering to the roots was used while the control soil was taken midway between rows. Samples were plated on a non-selective soil extract agar without added energy material (3), plates being held at 25° C. for two weeks. Colonies were systematically picked, approximately 180 for each sample, so that all on a plate or sector were taken, and stab inoculation made into soil extract semi-solid (containing 0.02% dipotassium hydrogen phosphate, 0.01% yeast extract, and 0.3% agar).

The nutritional grouping of the isolates was carried out by the procedure previously described (10) involving growth response in all of the following media:

Medium *B* —Basal medium
Medium *A* —Basal medium + amino acids
Medium *G* —Basal medium + growth factors
Medium *AG*—Basal medium + amino acids + growth factors
Medium *Y* —Basal medium + yeast extract
Medium *S* —Basal medium + soil extract
Medium *YS*—Basal medium + yeast extract + soil extract

Nutritional Groups in Rhizosphere and Control Soil

The relative incidence of the various nutritional groups of bacteria in the control and rhizosphere soils, and the estimated number of organisms of the different groups per gram of soil are shown in Table I. The outstanding

TABLE I

NUTRITIONAL GROUPS OF BACTERIA IN RHIZOSPHERE AND CONTROL SOIL

Group	Nutritional requirements for maximum growth	Control soil		Rhizosphere soil		Times increase in rhizosphere
		%	Number per gm.	%	Number per gm.	
	(Plate count)		37,500,000		532,000,000	14.2
I	Grow in basal medium	12.0	4,500,000	22.5	119,700,000	26.6
II	Require one or more amino acids	6.8	2,550,000	25.0	133,000,000	52.2
III	Require growth factors	23.1	8,660,000	15.0	79,800,000	9.2
IV	Require amino acids and growth factors	16.2	6,080,000	15.0	79,800,000	13.1
V	Require yeast extract	16.2	6,080,000	11.7	62,200,000	10.2
VI	Require soil extract	6.8	2,550,000	5.8	30,800,000	12.1
VII	Require yeast extract and soil extract	11.1	4,160,000	2.5	13,300,000	3.2

feature is the increased percentage, in the rhizosphere, of organisms requiring amino acids for maximum growth (Group II), as compared with the control soil. This preferential stimulation is also reflected in the total numbers of this group in the rhizosphere soil, representing a 52-fold increase as compared with a 14-fold increase in total numbers. The table also shows that those groups of bacteria that are dependent upon the more complex nutrients provided by yeast extract and soil extract (Groups V, VI, VII) and not furnished by the amino acids and growth factors, are relatively less abundant in the rhizosphere. This is specially true of those organisms that require for maximum growth certain unidentified substances in both yeast and soil extract (Group VII). On the other hand, those forms that are capable of maximum growth in the basal sugar–salts medium (Group I) are relatively more abundant in the rhizosphere.

Amino Acid Requirements

The relation of amino acid nutrition to the growth of bacteria in the rhizosphere and control soils is presented in greater detail in Table II. A comparison of the growth of the bacteria in the seven media indicated above permits, not only a grouping on the basis of maximum growth, as in Table I, but also a determination of the incidence of those organisms for which amino acids act as essential substances or as a stimulant for growth. In addition to those from the mangels experiment (1945), Table II gives results from earlier rhizosphere studies,* in so far as the relation to amino acids can be calculated from the data. In the earlier work all seven comparative media now employed were not used, but consisted in the 1941 series of Media *B*, *A*, and *G*, and in the 1939 tests of *B*, *A*, and *AG;* thus the same differentiation now possible could not be made.

* *Carried out with the co-operation of P. M. West (1939), and J. J. R. Campbell (1941).*

TABLE II

AMINO ACIDS IN NUTRITION OF BACTERIA IN RHIZOSPHERE AND CONTROL SOIL

—	Mangels (1945)		Flax (1941)		Flax (1939)		Tobacco (1939)	
	%		%		%		%	
	Control	Rhizo.	Control	Rhizo.	Control	Rhizo.	Control	Rhizo.
No growth or submax. growth in basal med.								
Max. growth with amino acids	6.8	25.0	8.4	17.3	4.3	16.5	11.8	13.6
Max. growth, cysteine alone	4.3	13.3	3.1	5.8	—	—	—	—
No growth in basal medium								
Growth (max. or submax.) with amino acids	20.5	30.0	7.3	11.0	14.9	29.1	14.0	24.0
Growth (max. or submax.) with cysteine	13.7	20.9	7.3	10.5	—	—	—	—
Submax. growth in basal medium								
Stimulated by amino acids	10.2	13.3	4.3	14.1	0	14.8	9.6	13.1
Stimulated by cysteine alone	6.0	8.3	4.3	8.8	—	—	—	—
Submax. growth in Medium *G*								
Stimulated by amino acids	17.9	20.8	—	—	—	—	—	—

From Table II it is seen that in all cases there is a preferential stimulation in the rhizosphere of those bacteria for which amino acids provide maximum growth, are essential for growth, or stimulate growth. This is believed to be one of the most characteristic rhizosphere effects as far as the nutritional characteristics of soil bacteria are concerned.

Growth Factor Requirements

Analogous data with respect to the growth factor nutrition of bacteria in rhizosphere and control soils are shown in Table III with the inclusion of

TABLE III

GROWTH FACTORS IN NUTRITION OF BACTERIA IN RHIZOSPHERE AND CONTROL SOIL

—	Mangels (1945)		Flax (1941)		Flax (1939)		Tobacco (1939)	
	%		%		%		%	
	Control	Rhizo.	Control	Rhizo.	Control	Rhizo.	Control	Rhizo.
No growth or submax. growth in Med. *B* or *A*								
Max. growth with growth factors	23.1	15.0	23.2	17.8	8.6	12.4	19.3	19.3
Max. growth, growth fact. and amino acids	16.2	15.0	—	—				
No growth in Med. *B* or *A*								
Growth (max. or submax.) with gr. factors	15.4	19.2	13.7	7.9	14.9	11.0	17.2	15.6
Growth (max. or submax.) with gr. factors and amino acids	7.7	5.0	—	—				
Submax. growth in Medium *B*								
Stimulated by growth factors	9.4	3.3	7.3	8.4	—	—	—	—
Submax. growth in Medium *A*								
Stimulated by growth factors	20.5	10.0	—	—	4.3	16.7	20.4	26.1

findings from earlier studies as far as the data permit calculation. In contrast to the results for amino acids, no significant 'rhizosphere effect' is noted with respect to the relative incidence of bacteria responding to the growth factors used.

Associative and Antagonistic Effects

Though the equilibrium between various groups of organisms existing in a soil at a given time will depend in large measure upon the availability of nutrients required for the growth of these organisms, associative and antagonistic effects are factors that might well play a role in establishing the microbial balance under a given set of conditions.

As noted in Table I, bacteria of Group I, capable of maximum growth in the basal medium, were relatively more numerous in the rhizosphere than in the control soil, though stimulated less than organisms of Group II. An attempt was made to study the action of Group I organisms on those of Groups II and III, respectively, by noting the effect of the culture filtrates of the first group on the growth of the other groups: (*a*) in the basal medium in which they normally do not grow, and (*b*) in Media *A* and *G*, respectively, to observe antibiotic effect in media in which they normally grow well.

Ten organisms of Group I were inoculated respectively into 50 ml. of the basal medium (*B*). After five days' incubation at 25° C. the cultures were filtered (Seitz) and 0.5 ml. of the respective filtrates added aseptically to different series of tubes each containing 5 ml. of Medium *B* or Medium *A*. Tubes containing filtrate from each of the Group I organisms, as well as control tubes without filtrate, were inoculated with one 1-mm. loopful of a suspension of each of 10 different strains of Group II organisms in respective series and incubated at 25° C. for five days. The tubes were then examined to note the effect of the different filtrates on the growth of each of the test organisms of Group II. Analogous series were prepared with Medium *B* and Medium *G* to note the effect of similar filtrates on Group III organisms.

The results, shown in Table IV, indicate that as far as the organisms tested are concerned, bacteria of Group I not only provide much greater stimulation to those of Group II than Group III, but are much more antagonistic towards Group III. This greater compatibility between Groups I and II than between Groups I and III is shown by a higher percentage of cases in which the filtrates permit Group II bacteria to grow in the basal medium (46.7% as compared with 18.2%), and by a much lower percentage of cases in which antibiotic effects were noted (4.4% as compared with 38.7%).

Discussion

The increased incidence in the rhizosphere of bacteria capable of maximum development in an amino acid medium or stimulated by amino acids raises an interesting speculation as to the relation of this to plant excretions, the exact nature of which is little understood. Though amino acids have been detected in soil and are regarded chiefly as products of the decomposition of organic

nitrogenous residues, the excretion of amino acids by plants was established by Virtanen and Laine (16) who identified aspartic acid and β-alanine in the nitrogenous excretions of leguminous plants. The environmental factors determining such excretion have been thoroughly investigated by Wilson and associates (18). That amino acids may be excreted by non-leguminous plants in amounts sufficient to modify the balance of nutritional groups of bacteria may be postulated.

The formation of amino acids as decomposition products of sloughed-off portions of roots also suggests itself as a possible factor in the rhizosphere effect on the amino acid group of bacteria. Excretion by the root appears more likely, however, inasmuch as the effect may be exerted by the plant in the seedling stage, as was the case in the 1939 and 1941 experiments with flax (Table II).

TABLE IV

ASSOCIATIVE AND ANTAGONISTIC ACTION OF GROUP I ORGANISMS ON BACTERIA REQUIRING AMINO ACIDS AND GROWTH FACTORS (GROUPS II AND III)

Filtrates of Group I bacteria* (Cult. No.)	Group II bacteria**				Group III bacteria***			
	No. of strains tested	No. able to grow in basal med. + filtrate	No. suppressed by filtrate in Med. *A*		No. of strains tested	No. able to grow in basal med. + filtrate	No. suppressed by filtrate in Med. *G*	
			Partially	Completely			Partially	Completely
*C*77	10	3	2	0	11	0	4	0
*R*10	—	—	—	—	11	5	3	1
*R*14	10	4	1	0	11	1	3	4
*R*34	10	2	1	0	—	—	—	—
*R*36	10	3	0	0	11	1	3	1
*R*52	10	8	0	0	11	0	4	2
*R*85	10	5	0	0	11	3	4	0
*R*86	10	6	0	0	11	1	3	2
*R*87	10	6	0	0	—	—	—	—
*R*115	10	5	0	0	11	5	0	0
		% 46.7	% 4.4	% 0		% 18.2	% 27.3	% 11.4
			4.4				38.7	

* *Maximum growth in basal medium.*
** *No growth in basal medium; require amino acids.*
*** *No growth in basal medium; require growth factors.*

The data in Table IV suggest that the preferential stimulation of the amino acid group may be concerned, at least partially, with the phenomenon of associative action between different groups of organisms. This influence, however, may be related indirectly to a shifting of the bacterial equilibrium due to plant excretion, for instance, through a stimulation of Group I bacteria, which might in turn stimulate Group II.

The data here presented furnish indirect rather than direct evidence regarding the mechanism by which the development of special groups of soil bacteria

in the rhizosphere is induced. However, the study of nutritive differences in micro-organisms in the rhizosphere is offered as one useful means of approach in obtaining a better knowledge of the physiological activity of the root system of plants, and of factors concerned with such practical problems as crop rotation and the control of soil-borne diseases of crops.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS: VIII. INFLUENCE OF VARIOUS CROP PLANTS ON THE NUTRITIONAL GROUPS OF SOIL BACTERIA[1]

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The study of different physiological groups of soil bacteria has been concerned largely with observations on organisms developing on media selected to favor such groups. Mainly because of a lack of suitable methods, relatively little is known of a large proportion of the microorganisms indigenous to soil. In this series of investigations an attempt has been made to develop a more objective approach to the study of soil bacteria, in which emphasis is placed on the organisms themselves, isolated by nonselective methods and the functions of which are unknown, rather than on special groups of soil organisms associated with various known biochemical processes (7, 13).

Based upon the assumption that the relative abundance of soil microorganisms depends mainly upon availability of nutrients necessary for their growth, a method of grouping soil organisms has been proposed (9). This depends upon the determination of growth response in a series of media of increasing nutritional complexity. It is felt that the application of this method of study to an investigation of microorganisms of the rhizosphere, that is, the immediate zone of influence of the living plant root, might contribute to a better knowledge of the nature of root excretions and of factors related to crop rotation and the control of soil-borne diseases of crops (10).

Many previous investigations, beginning with the observations of Hiltner (3), have established the fact that soil in the rhizosphere contains higher numbers of microorganisms than soil not within the influence of the plant root. It has been shown also that in the rhizosphere the balance between certain physiological groups is changed (1, 5, 11, 12) as well as that between different morphological types of bacteria or fungi (6, 8, 14). Studies with respect to nutritional groups of soil bacteria have shown a preferential stimulation of certain of these groups in the rhizosphere soil as contrasted with soil not under the influence of the growing plant root (4, 10, 17).

The purpose of the present investigation was to compare different crop plants, studied at different growth stages, to note any specific rhizosphere effects which may be exerted in modifying the balance between different nutritional groups of soil bacteria.

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MATERIALS AND METHODS

Six crops—wheat, oats, red clover, timothy, alfalfa, and flax—were greenhouse grown in soil obtained from a plot in a 4-year rotation system of oats, clover, timothy, and mangels, 15 tons of manure being applied to mangels. A series of pots with uncropped soil served as controls. Duplicate samples for analysis were removed at two periods of plant development, one at the seedling stage and the other at or near the flowering stage. To obtain the rhizosphere samples, several plants, varying with the crop, were carefully taken from the soil, the roots shaken briskly to remove as much of the adhering soil as possible and then placed in 100 ml. of sterile water in weighed flasks. Sufficient roots were added to approximate the turbidity of the 1:100 dilution of the control sample. Appropriate dilutions of both control and rhizosphere samples were made and aliquots of the final dilutions were plated with soil extract agar (13). The roots were removed from the flasks in which the original suspensions were made, and the contents, as well as those with the control soil suspensions, were evaporated to dryness for the estimation of plate counts on a dry-weight basis, correction being made for the aliquots removed.

All plates were incubated at 25°C. for 10 to 14 days. From the whole area of a plate on which the colonies were suitably dispersed or from a definite sector of a plate, all colonies were systematically picked off until approximately 100 had been removed; these were cultivated as stab cultures in soil extract semisolid agar (9) to serve as stock cultures for further study.

The differential media and methods of grouping described by Lochhead and Chase (9) were used for determining the nutritional requirements of the isolated organisms; accordingly, the various bacteria were divided into the following groups:

Group I—Bacteria with simple requirements.
Group II—Bacteria requiring one or more amino acids.
Group III—Bacteria requiring growth factors.
Group IV—Bacteria requiring amino acids plus growth factors.
Group V—Bacteria requiring unidentified substances in yeast extract.
Group VI—Bacteria requiring unidentified substances in soil extract.
Group VII—Bacteria requiring unidentified substances in both yeast and soil extract.

GENERAL RHIZOSPHERE EFFECTS

Table 1 shows the total plate count for each sample as well as the percentage incidence of each nutritional group from the rhizospheres of the seedling and older plants respectively, compared with the corresponding groups of bacteria in the control soil. The percentage values were calculated from the total numbers of cultures obtained from the two replicates of each soil and rhizosphere sample.

The results indicate that for all the crop plants there is, in the rhizosphere, a larger proportion of bacteria with relatively simple nutritional requirements as compared with the control soil. This is shown by the preferential stimulation of organisms capable of maximum growth in the basal medium and of those

requiring amino acids (groups I and II), and in the case of older plants, of those responding to growth factors (group III). On the other hand, bacteria requiring for maximum growth the more complex substances in yeast and soil extract, particularly groups VI and VII, are proportionately less abundant in the rhizosphere. The rhizosphere effect on the relative incidence of organisms of group V, requiring yeast extract for maximum growth, varied with the age of the plant. With the seedling plants this group was relatively decreased in the rhizosphere, with the exception of flax, whereas in the rhizosphere of older plants it showed a proportionate increase throughout.

TABLE 1

Plate counts and percentage incidences of nutritional groups for control soils and rhizosphere soils of various crop plants

	WHEAT		OATS		RED CLOVER		TIMOTHY		ALFALFA		FLAX	
	C*	Rh.*	C	Rh.	C	Rh.	C	Rh.	C	Rh.	C	Rh.
Seedling Plants												
Plate count..... *millions/gm.*	123	502	250	2080	134	3225	134	826	134	825	199	1119
Group I—Basal medium.....%	10.4	37.7	12.6	33.1	6.8	34.6	6.8	21.8	6.8	29.1	7.3	26.0
Group II—Amino acids.....%	4.0	4.6	2.2	11.0	2.5	20.9	2.5	4.5	2.5	11.4	2.4	9.8
Group III—Growth factors....%	12.8	20.0	8.9	11.7	11.9	13.1	11.9	11.7	11.9	14.3	8.5	12.7
Group IV—Amino acids growth factors.....%	0.8	3.0	1.5	0.0	9.3	9.2	9.3	8.9	9.3	10.3	7.3	5.2
Group V—Yeast extract.....%	21.6	17.7	26.7	18.6	19.5	11.1	19.5	14.0	19.5	14.9	10.3	30.0
Group VI—Soil extract.....%	31.2	12.0	23.0	17.9	37.3	8.5	37.3	29.0	37.3	15.4	26.0	12.1
Group VII—Yeast extract soil extract.....%	17.6	3.4	24.4	7.6	12.7	3.3	12.7	9.5	12.7	4.0	37.6	4.1
Older Plants												
Plate count..... *millions/gm.*	199	1338	199	1275	257	3872	257	837	257	3927	146	1691
Group I—Basal medium.....%	7.3	30.3	7.3	25.0	2.4	31.1	2.4	10.8	2.4	21.7	4.3	23.5
Group II—Amino acids.....%	2.4	18.2	2.4	22.4	0.0	11.7	0.0	4.6	0.0	15.5	4.3	9.4
Group III—Growth factors....%	8.5	16.7	8.5	10.9	1.6	15.3	1.6	16.4	1.6	14.0	0.9	21.2
Group IV—Amino acids, growth factors.....%	7.3	4.2	7.3	5.1	1.6	15.3	1.6	3.1	1.6	3.1	2.6	2.3
Group V—Yeast extract.....%	10.3	17.0	10.3	23.1	7.1	14.6	7.1	13.2	7.1	18.6	12.8	18.8
Group VI—Soil extract.....%	26.0	7.9	26.0	5.1	42.0	13.1	42.0	34.9	42.0	14.7	35.0	16.5
Group VII—Yeast extract soil extract.....%	37.6	4.8	37.6	7.9	46.0	15.3	46.0	17.8	46.0	12.4	39.3	8.2

* C = Control; Rh = Rhizosphere.

SPECIFIC RHIZOSPHERE EFFECTS OF VARIOUS CROP PLANTS

To note whether specific rhizosphere effects are exerted by the different crop plants, an analysis of variance was calculated separately for each bacterial group. The numbers of organisms per gram of dry soil representing the respective groups were calculated from the total plate count and the percentage incidences for each sample. These values were transferred to logarithms for use in the analysis of variance, in line with the recommendations of Cochran (2). The different sources of variation and their respective mean variances are shown in table 2.

The soils-crop plants interaction (specific rhizosphere effect) indicates significant differences in rhizosphere effect between the crops with respect to bacterial

groups I, II, III, IV, and V. These differences may be judged from graphs of the data used for calculation of the respective variances. Figure 1 depicts, for each group affected, differences between the logarithms of the rhizosphere soil numbers and those of control soils.

On the basis of the greatest differences between the various crops, it appears that red clover and alfalfa have a higher rhizosphere effect than wheat, oats, and timothy with respect to group I bacteria. For group II organisms, the effects of the rhizospheres of red clover and alfalfa are different from those of wheat, timothy, and flax. For group III organisms the effects of red clover,

TABLE 2

Values calculated from the analysis of variance for each nutritional group

SOURCE OF VARIATION	DEGREES OF FREEDOM	MEAN VARIANCES						
		Group I	Group II	Group III	Group IV	Group V	Group VI	Group VII
Age of plants	1	0.06	0.14	0.07	0.00	0.01	0.09	2.46
Soils (control and rhizosphere)	1	31.15	31.15	19.39	10.14	11.66	3.77	1.67
Crop plants	5	0.29	0.32	0.13	0.50	0.19	0.39	0.28
Age of plants—soils	1	0.03	0.29	1.09	0.10	0.41	0.01	0.01
Age of plants—crop plants	5	0.11	0.11	0.12	0.52	0.10	0.25	0.40
Soils—crop plants (specific rhizosphere effect)	5	0.24*	0.37**	0.29**	0.34**	0.17**	0.06	0.07
Age of plants—soils—crop plants (effect of age)	5	0.09	0.26**	0.41**	0.16*	0.04	0.23*	0.03**
Error	24	0.07	0.04	0.02	0.05	0.03	0.06	0.03
Total	47	36.64	37.81	25.90	18.89	15.31	9.99	10.15

* = Significant.
** = Highly significant.

alfalfa, and flax differ from those of wheat and oats. In bacterial group IV, the rhizosphere of red clover is different from those of all the other plants with the possible exception of alfalfa. In Group V, the effects of red clover, alfalfa, and flax are significantly greater than those of wheat, oats, and timothy. In general, the legume crops show a greater effect than the cereals and timothy, but only in two groups do they appear to have a different effect from that of flax.

RHIZOSPHERE EFFECT IN RELATION TO AGE OF PLANT

Calculations with respect to the age of plants-soils-crops interaction (age-rhizosphere effect) indicate that the age of certain crops significantly alters the bacterial population representing groups II, III, IV, VI, and VII. Figure 2 shows graphically the rhizosphere and control relationships of seedling plants compared with those of older plants. During the growth of wheat and alfalfa the rhizosphere effects appear to increase significantly with respect to group II

organisms. Likewise, the effects of alfalfa and flax increase for group III bacteria. In group IV organisms, increases are apparent with respect to the rhizosphere effects of red clover and flax, whereas that of wheat seems to have decreased. For group VI bacteria, the influence of the older wheat rhizosphere is greater than that of the seedling plant, whereas the reverse appears to be true for timothy. Finally, the effects of the older red clover and timothy rhizo-

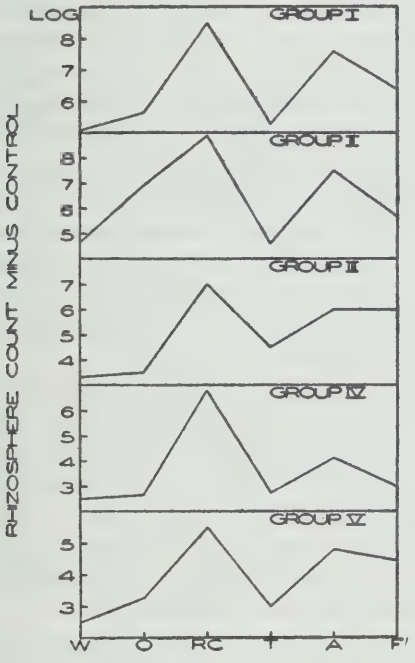

FIG. 1

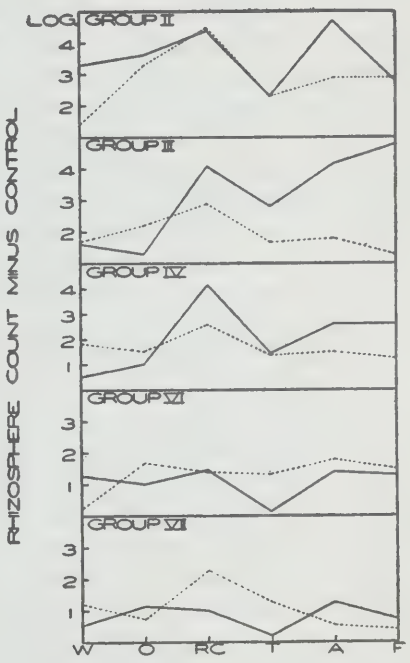

FIG. 2

FIG. 1. SPECIFIC RHIZOSPHERE EFFECT OF VARIOUS CROP PLANTS ON NUMBERS OF DIFFERENT NUTRITIONAL GROUPS OF BACTERIA

W = wheat; O = oats; RC = red clover; T = timothy; A = alfalfa; F = flax

FIG. 2. RHIZOSPHERE EFFECT IN RELATION TO AGE OF VARIOUS CROP PLANTS

W = wheat; O = oats; RC = red clover; T = timothy; A = alfalfa; F = flax. Solid line = older plants; broken line = seedling plants.

spheres appear to be significantly diminished with respect to group VII organisms.

DISCUSSION

In soil there is increasing evidence of a microbiological equilibrium, though one which is always subject to change through the influences of season, temperature, moisture, soil treatment, and cropping. Of these factors, which affect not only the numbers but also the balance between different groups of soil bacteria, the growing plant appears to have the greatest effect under normal conditions. Previous studies on the influence of the growing plant on soil bacteria, by use of

the quantitative-qualitative approach on a nonselective basis, have indicated a rhizosphere effect characteristic of all plants studied. In the zone of influence of the root there has been noted a qualitative change in the bacterial flora, shown by a shifting of the balance between different groups of organisms, whether these are classified on the basis of morphology, general physiological activity, or, by what is believed to be a more rational grouping, according to their nutritional requirements.

Though different crop plants conform to a general pattern in their rhizosphere effect on soil bacteria, results of the present study point to specific effects exerted by different crops in modifying the microbial balance in soil. Thus legumes, particularly, appear to exert a more pronounced effect in stimulating significantly bacteria of groups I, II, and, in the case of red clover, group IV. Since groups II and IV both require amino acids, this finding is of interest in connection with the demonstration of amino acid excretion by leguminous plants (15). A further point of interest is the effect of flax which, together with the two legumes, has a more pronounced influence than wheat, oats, or timothy on organisms of groups III and V, requiring respectively known growth factors or those contained in yeast extract. This finding supports indirectly the work of West (16) respecting the excretion of thiamin and biotin by the roots of flax seedlings.

It is recognised that the preferential stimulation of certain microbial groups in the rhizosphere cannot necessarily be attributed to the effect of root excretion alone, but that decomposition products of sloughed-off epidermal cells and root hairs must be considered. Since fewer significant differences in rhizosphere effects were noted, however, between seedling and older plants than between the crops themselves, the results suggest that the changes in the microbial balance in the rhizosphere cannot be ascribed solely to the breakdown of sloughed-off material, and that root excretion must be considered as an important factor.

The indication that different crop plants exert specific effects on the bacterial flora, by which the qualitative nature of the organisms in the environment of the growing plant is altered, points to the value of further work to elucidate more fully such specific influences. This not only will contribute to a greater knowledge of root physiology, but should aid in a better understanding of the fundamentals underlying the principles of crop sequence and related questions of plant growth, as well as plant disease problems related to soil conditions.

SUMMARY

Bacteria isolated from the rhizospheres of wheat, oats, red clover, timothy, alfalfa, and flax, as well as from uncropped soils, were grouped according to their nutritional requirements.

A general rhizosphere effect shown by all crops at two stages of growth was indicated by a much higher percentage, in the rhizosphere, of bacteria with the simplest nutritional requirements than in soil without crops. The situation was reversed with regard to bacteria requiring the complex nutrients of soil extract.

Significant differences in rhizosphere effect between certain crops were noted for bacteria giving maximum growth response in a glucose-inorganic-salts medium and in that medium supplemented with amino acids, growth factors, amino acids

and growth factors, and yeast extract respectively. In the main, these specific differences existed between red clover, alfalfa, and flax on the one hand and wheat, oats, and timothy on the other. The legumes, however, differed from flax with respect to bacteria responding to amino acids and amino acids plus growth factors.

Significant differences were found in the rhizosphere effect on five of the bacterial groups between plants at the seedling and flowering stages, but such differences were limited, in the case of each group, to one or two crops.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS

IX. AMINO ACID REQUIREMENTS OF RHIZOSPHERE BACTERIA[1]

By R. H. Wallace[2] and A. G. Lochhead[3]

Abstract

A study was made of the more specific amino acid requirements of bacteria from the rhizospheres of clover, flax, and wheat plants for which a chemically defined medium containing 23 amino acids provided essentials for maximum growth. Of seven groups of amino acids, the sulphur-containing group (cysteine, methionine, and taurine) was found to be of special significance, the omission of this group resulting in a pronounced decrease in the percentage of organisms able to develop. Further study of organisms dependent upon this group of amino acids for growth showed methionine to be by far the most essential compound. While evident for bacteria from the rhizosphere of all three crops, the effect was more pronounced in the case of clover than with flax or wheat.

Introduction

One of the important groups into which soil bacteria may be divided on the basis of nutritional requirements comprises those organisms for which amino acids are needed for growth (3). This group appears to be significantly related to crop growth, for studies with a variety of plants have shown that bacteria requiring amino acids are preferentially stimulated in the rhizosphere (4, 6).

In the procedure proposed in a previous paper of this series (3) for classifying soil bacteria according to their nutritional requirements, the medium adopted for organisms requiring amino acids consisted of a basal glucose–nitrate–salts medium with the addition of 10 amino acids. However, it would be expected that a medium containing a larger number of amino acids, some of which would doubtless be required by bacteria not responding to the 10 amino acids previously selected, would increase the percentage of organisms falling within the 'amino acid group'. The original medium, however, has provided a basis for grouping that has been found helpful in studies of the microbial equilibrium in soil as affected by plant growth or other environmental conditions (2, 4, 6) and in determining the 'bacterial balance index' in relation to certain plant disease factors in soil (1, 5).

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Within any given 'nutritional group' there may be considerable divergencies, not only in morphological type, but with respect to the individual nutritional requirements of species or strains. Previous studies (3) of a series of isolates from soil, all responding to a mixture of amino acids, showed wide differences in response to single amino acids, the results suggesting that indigenous soil bacteria may exercise highly specialized functions, and indicating incidentally the high degree of variation between many apparently closely related types.

The purpose of the studies here reported was to examine more specifically the amino acid requirements of bacteria isolated from the rhizosphere and to ascertain which compounds may be responsible for the increase of organisms of the 'amino acid group' within the zone of influence of the growing plant root.

Experimental

Bacteria were isolated from the rhizospheres of field-grown clover, flax, and wheat. The respective plants were brought from the field to the laboratory and, after superfluous quantities of soil had been carefully removed, the roots and adhering soil were placed in sterile water, shaken, and suitable dilutions made for plating. Soil extract agar without added energy material was used for plating as being the least selective medium available. It was prepared by autoclaving 1 kgm. garden or field soil with 1 liter tap water for 30 min. at 15 lb., filtering after a little calcium sulphate was added, and making the filtrate up to 1 liter. To the soil extract were added 0.02% potassium monohydrogen phosphate and 1.5% agar. Plates were incubated at 25° C. for 12 days. All colonies that developed were picked off from the whole area of suitable plates or sectors (approximately 300 from each sample) and stab inoculations made into soil extract semisolid medium (containing 0.02% potassium monohydrogen phosphate, 0.01% yeast extract, 0.3% agar) for further study.

To determine which of the isolated organisms required amino acids for good growth, transfers were made to two fluid media, namely a basal glucose–salts medium with potassium nitrate as the only source of nitrogen (3), and this medium supplemented with the following 23 amino acids each at 0.025% concentration.

dl-Alpha alanine
l-Arginine monohydrochloride
l-Asparagine
dl-Aspartic acid
l-Cysteine hydrochloride
glycine
l-Glutamic acid
l-Histidine monohydrochloride
l-Hydroxyproline
dl-Isoleucine
l-Leucine
dl-Lysine monohydrochloride
dl-Methionine
dl-Norleucine
dl-Ornithine monohydrochloride
dl-Phenylalanine
l-Proline
dl-Serine
l-Taurine
dl-Threonine
l-Tryptophane
l-Tyrosine
dl-Valine

Organisms showing no growth in the basal medium and good growth in the presence of amino acids after five days' incubation at 25° C. (197 cultures) were selected for study and transferred to a series of seven media in which the following groups of amino acids were respectively omitted. The grouping was made on the basis of related characteristics of the formulae ascribed to the respective compounds.

Group 1. Alanine, glycine, norleucine, serine, threonine.
Group 2. Cysteine, methionine, taurine.
Group 3. Isoleucine, leucine, valine.
Group 4. Aspartic acid, glutamic acid.
Group 5. Arginine, asparagine, lysine, ornithine.
Group 6. Histidine, hydroxyproline, proline.
Group 7. Phenylalanine, tryptophane, tyrosine.

Growth Responses to Amino Acid Groups

Table I shows the effect of omitting different groups of amino acids on the growth of bacteria capable of maximum development in the complete amino acid medium and showing no growth in the basal medium. It is apparent that there is considerable diversity with respect to the amino acids needed by certain bacteria of the rhizosphere microflora for growth, since in all cases some forms were unable to develop in the absence of each group of amino acids. However, though in the absence of the amino acids of Group 1 a somewhat higher percentage of organisms failed to grow, Group 2, comprising cysteine, methionine, and taurine, exerted the most significant effect, its omission resulting in a pronounced increase in the percentage of bacteria showing no growth or submaximal growth, and a corresponding striking decrease in the percentage capable of maximum growth. The effect was similar in the case of all three crops.

The Sulphur-containing Amino Acids

Bacteria showing maximum growth in the complete amino acid medium and no growth in the absence of sulphur-containing amino acids (95 cultures) were selected for further observation. To determine which of the three compounds in Group 2 caused the differences noted in Table I, these cultures were transferred to media having the full complement of amino acids but with the following respective omissions:

1. Cysteine.
2. Methionine.
3. Taurine.
4. Cysteine and methionine.
5. Cysteine and taurine.
6. Methionine and taurine

TABLE I

EFFECT OF OMITTING DIFFERENT GROUPS OF AMINO ACIDS ON GROWTH OF BACTERIA FROM RHIZOSPHERES OF CLOVER, FLAX, AND WHEAT

Medium	Composition of medium	—	Source of bacteria (rhizosphere)		
			Clover	Flax	Wheat
			Percentage of bacteria		
B	Control—basal (no amino acids)	Growth	0.0	0.0	0.0
A	Control—basal + 23 amino acids	Max. growth	100.0	100.0	100.0
A−1	A minus alanine, glycine, norleucine, serine, threonine	Max. growth	67.8	60.0	49.0
		Submax. "	9.6	21.2	10.6
		No "	22.6	18.8	40.4
A−2	A minus cysteine, methionine, taurine	Max. growth	29.0	18.8	19.2
		Submax. "	29.1	30.6	31.9
		No "	41.9	50.6	48.9
A−3	A minus isoleucine, leucine, valine	Max. growth	87.1	75.3	66.0
		Submax. "	6.4	14.1	17.0
		No. "	6.5	10.6	17.0
A−4	A minus aspartic acid, glutamic acid	Max. growth	71.0	81.1	74.5
		Submax. "	25.8	13.0	23.4
		No "	3.2	5.9	2.1
A−5	A minus arginine, asparagine, lysine, ornithine	Max. growth	74.2	82.4	72.4
		Submax. "	16.1	10.5	23.3
		No "	9.7	7.1	4.3
A−6	A minus histidine, hydroxyproline, proline	Max. growth	90.3	87.0	80.8
		Submax. "	6.5	8.3	19.2
		No "	3.2	4.7	0.0
A−7	A minus phenylalanine, tryptophane, tyrosine	Max. growth	87.1	74.2	66.0
		Submax. "	9.7	11.7	17.0
		No "	3.2	14.1	17.0

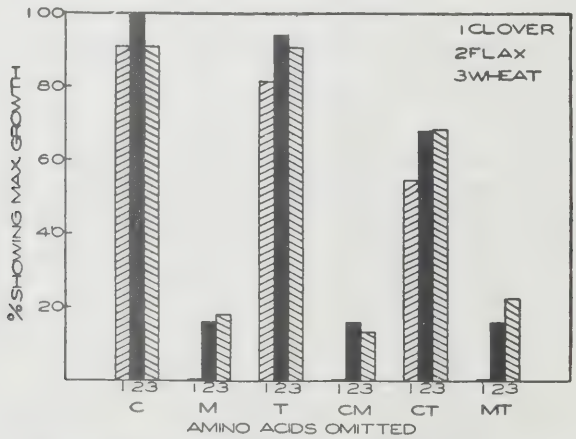

FIG. 1. *Effect of omitting cysteine (C), methionine (M), and taurine (T) singly and in combinations on growth in amino acid medium of rhizosphere organisms from three crop plants.*

As shown in Fig. 1, the absence of methionine resulted in a remarkable reduction in the number of cultures producing maximum growth. Though this effect appears to be common to the organisms from all three crops, it is more pronounced in the case of clover than that of wheat and flax. It also seems that notably more cultures were capable of maximum growth in the presence of methionine plus cysteine or taurine than with methionine as the only sulphur-containing amino acid.

Discussion

Previous investigations have pointed to the fact that the rhizosphere is a unique zone, where conditions are such as to modify considerably the bacterial equilibrium normally existent in a soil of a given type. The increased incidence in the rhizosphere of bacteria giving growth response to amino acids has provided evidence of interest, though indirect, with regard to the problem of root excretions and has directed attention consequently to a more detailed study of the specific nutrient requirements of such organisms. Results of the work reported here indicate that, for the most part, rhizosphere bacteria dependent upon amino acids for growth are remarkably versatile in their ability to utilize the various amino acids. It appears that the respective omission of each group of amino acids, with one exception, has little effect on the ability of the majority of cultures to produce good growth. The nitrogen value, therefore, of each group omitted would appear to be readily replaceable by that of the other groups remaining in the media.

In view of the foregoing it would seem probable that the sulphur-containing group of amino acids, the one group whose omission resulted in but a comparatively small number of cultures being able to show maximum growth, may serve in some other physiological role besides that of supplying nitrogen. The most important member of this group appears to be methionine, a substance possibly useful because of a mobile methyl group, either in the synthesis of an essential metabolite or as a more readily available source of sulphur.

The relatively high percentage of organisms incapable of growing in artificial media in which methionine was omitted, suggests that this amino acid is made available in the environment from which the organisms were isolated originally, and intensifies the value of further work to elucidate more directly the role of various factors concerned with its occurrence, including excretion by roots, formation by decomposition of plant tissue, or elaboration by soil organisms of other nutritional groups.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS

X. BACTERIA REQUIRING VITAMIN B_{12} AS GROWTH FACTOR[1]

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Studies of the nutritional requirements of soil bacteria isolated on a nonselective basis (Lochhead and Chase, 1943) have shown the presence of a well-defined group of organisms dependent on soil extract for maximum growth. Some of these bacteria show no growth; others at best, submaximal growth in media containing sugars and inorganic salts, supplemented by yeast extract or combinations of amino acids and vitamins (not including B_{12}). Though the relative incidence of this group may vary with the soil type, the chief factor affecting the proportion of these organisms in soil is the growing plant. Investigations with a variety of crops (Lochhead and Thexton, 1947; Wallace and Lochhead, 1949) have shown a lowered relative incidence in the rhizosphere, attributable to a preferential stimulation by the plant root of bacteria with simpler nutritional needs. However, beyond the influence of the roots, this group has been found to comprise from 8 to 35 per cent of the bacteria in various soils examined.

The present paper deals with a more detailed study of organisms, incapable of growth in yeast extract medium but showing good growth on addition of soil extract, following a preliminary report (Lochhead and Thexton, 1951) that for many of these bacteria vitamin B_{12} may replace the growth factor effect of soil extract.

MATERIAL AND METHODS

Bacteria were isolated from a field soil by plating on soil extract agar without added energy material, chosen as being the least selective medium for the development of the indigenous bacterial flora. It was prepared by autoclaving 1 kg field soil with 1 liter tap water for 30 min at 15 lb, filtering after addition of a little $CaSO_4$, and making the filtrate up to 1 liter. To the filtrate were added 0.02 per cent K_2HPO_4 and 1.5 per cent agar, with final pH of 6.8. Plates were incubated at 25 C for 12 days. From suitable plates or sectors all colonies were picked, and stab inoculations were made into soil extract semisolid (containing 0.02 per cent K_2HPO_4, 0.1 per cent yeast extract, 0.3 per cent agar) as stock cultures for further study.

The organisms isolated were differentiated according to nutritional requirements by the method of grouping described by Lochhead and Chase (1943), whereby growth response is determined in media of increasing complexity, ranging from a simple basal medium of glucose and inorganic salts to a complex

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medium including both yeast extract and soil extract. Special attention was given to bacteria requiring soil extract for maximum growth, but showing no growth in yeast extract medium. These organisms represent, therefore, a portion of Groups VI and VII of Lochhead and Chase (1943), which groups include those requiring soil extract for maximum growth, but showing no growth or submaximal growth in its absence.

Of 534 strains isolated from soil, 75 strains (14 per cent) were incapable of growth in yeast extract medium, though able to grow well upon the addition of soil extract. It had been suggested by Lochhead and Chase (1943) that soil extract contains bacterial growth-promoting factors not present in yeast extract or provided in the growth factor media used. Since vitamin B_{12} was not recognized at the time of the previous work, it was considered a matter of interest to determine (1) the ability of this vitamin to replace soil extract as growth-promoting factor, and (2) the ability of other soil organisms, not dependent upon soil extract, to produce the growth-promoting factor and the relation of this ability to their capacity for synthesizing vitamin B_{12}.

Media and Culture Filtrates

Yeast extract medium (medium Y): glucose, 1.0 g; K_2HPO_4, 1.0 g; KNO_3, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 0.1 g; NaCl, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; distilled water, 1 liter (basal medium); yeast extract, 1.0 g.

Yeast + soil extract medium (medium YS): 750 ml basal glucose salts medium; 250 ml soil extract, prepared as above; yeast extract, 1.0 g.

Yeast + vitamin B_{12} medium (medium YB_{12}): medium Y, as above, plus vitamin B_{12} to give a concentration of 2 mμg per ml (prepared from crystalline vitamin B_{12}).

Yeast + cobalt medium (medium YCo): medium Y, as above, plus 0.008 g $CoCl_2 \cdot 6H_2O$ per liter (to give a concentration of 2 μg Co^{++} per ml).

Yeast + culture filtrate media (media YF): medium Y, as above, plus 10 per cent of culture filtrate of the organism to be tested for growth factor production, prepared as indicated below.

Culture filtrates: To test for growth factor synthesis, bacteria not requiring soil extract were inoculated into 200 ml flasks containing 60 ml of a medium consisting of glucose, 10 g; K_2HPO_4, 1.0 g; KNO_3, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; $FeCl_3 \cdot 6H_2O$, 0.01 g; $CaCO_3$, 0.1 g; $CoCl_2 \cdot 6H_2O$, 0.008 g; casamino acids, 5.0 g; yeast extract, 1.0 g; distilled water, 1 liter (pH = 7.2). Cultures were shaken for 6 days at 26 C, adjusted to pH 4.0, heated for 6 min at 15 lb to destroy cells and to liberate bound vitamin B_{12}, filtered through paper, or Seitz filter if necessary, until clear, and sterilized. The filtrates were assayed for vitamin B_{12} content by the *Lactobacillus leichmannii* method, as well as used for preparing the yeast culture filtrate media (media YF) indicated above.

The media were tubed in 5 ml amounts and transfers made by 1 mm loop inoculation from the stock cultures. Cultures were incubated for 5 days at 25 C and then examined for growth response.

RESULTS

Results of preliminary tests on the ability of vitamin B_{12} to replace soil extract as growth promoting factor showed that of 75 strains requiring soil extract for growth, 41 were able to grow upon substitution of B_{12} (2 mμg per ml) for soil extract, 20 strains showing optimum, and 21 submaximal growth. Since the soil extract used had a vitamin B_{12} content of 1.96 mμg per ml (corresponding to a value of 0.49 mμg per ml in the soil extract medium YS), the findings support the view that the growth-promoting capacity of soil extract, for an important proportion of bacteria for which it is essential, depends upon the presence of vitamin B_{12}.

The occurrence of vitamin B_{12} in soil may be ascribed chiefly to two factors, (a) the application of organic manures of animal origin, and (b) synthesis of the

TABLE 1

Effect of vitamin B_{12} concentration on growth of 30 test organisms

B_{12} added mμg/ml	Test organisms showing growth	Degree of turbidity						Average turbidity score
		4	3	2	1	tr.*	0	
10.0	30	27	3	0	0	0	0	3.90
1.0	30	27	3	0	0	0	0	3.90
0.1	30	6	12	11	1	0	0	2.77
0.01	24	0	0	2	14	8	6	0.73
0.001	6	0	0	0	1	5	24	0.12
Controls								
Medium Y	2	0	0	0	0	2	28	0.03
Medium YCo	2	0	0	0	0	2	28	0.03
Medium YS	30	29	1	0	0	0	0	3.97

* Trace of growth, given arbitrary value of 0.5 in computing of turbidity score.

vitamin by microorganisms. Production of vitamin B_{12}-active substance has been shown by organisms from various sources, including soil. Robbins *et al.* (1950) have stated that as many as 50 per cent of the organisms developing on plates seeded with soil were found to produce vitamin B_{12}. Employing special selective media, Burton and Lochhead (1951) found 70 per cent of the bacteria isolated from local cultivated soil, and 84 per cent of the bacteria as well as 66 per cent of the actinomycetes from uncultivated soils of northern Canada to be capable of synthesizing vitamin B_{12}-active substances.

The effect of varying concentrations of vitamin B_{12} in promoting growth was studied in a series of experiments with 30 test organisms isolated from soil for which a concentration of 2 mμg per ml of the vitamin (medium YB_{12}) was nutritionally equivalent to soil extract (medium YS). Vitamin B_{12} was added to medium Y to give concentrations of 10.0, 1.0, 0.1, 0.01, and 0.001 mμg per ml of culture solution, respectively. As shown in table 1. good growth, approximating that provided by soil extract, was given by 1.0 mμg B_{12} per ml with no increase

at 10.0 mμg per ml. All test strains grew, though less profusely, at 0.1 mμg per ml while at lower concentrations growth diminished noticeably so that at 0.001 mμg per ml the great majority of the cultures showed no perceptible development.

Since vitamin B_{12} is known to contain cobalt, an element therefore necessary for its synthesis and occurring in varying amounts in soils, the possibility that the growth-promoting effect of soil extract might be due to its cobalt content, thus permitting synthesis of the vitamin by the test organisms, was examined by noting the effect of Co^{++} (2 μg per ml) added to medium Y. However, as shown

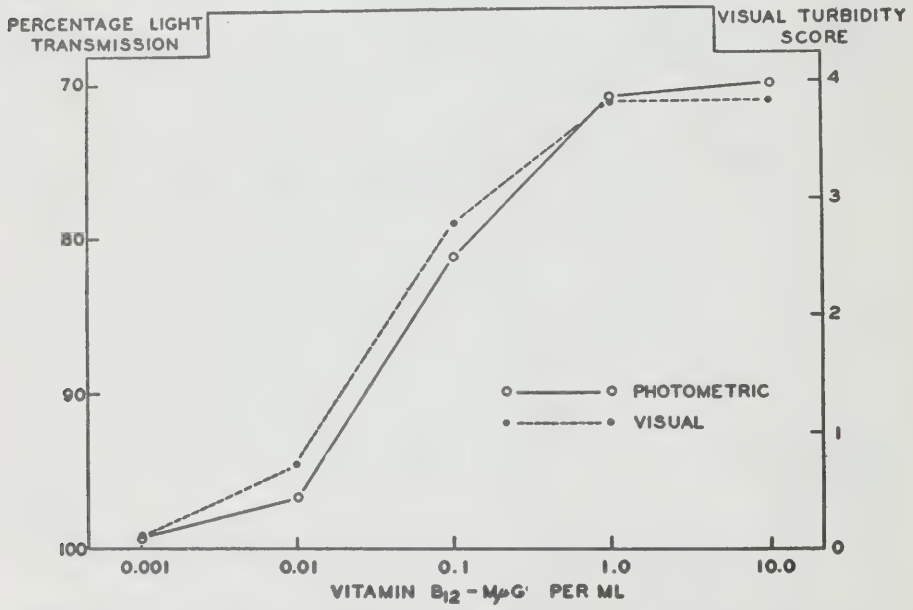

Figure 1. The effect of vitamin B_{12} concentration on the growth of 16 type cultures. Comparison of measurements (average) made by photometric and by visual turbidity readings.

in table 1, the addition of cobalt was without effect, indicating that in the nutrition of the test bacteria the cobaltous ion was unable to replace the vitamin.

In the case of 16 cultures, representing the widest variety of bacterial types in the group under study, readings were made first by visual observation and then by means of a photoelectric turbidimeter. As indicated in figure 1, giving the average values from the two methods of estimating growth, the most significant range of effectiveness of B_{12} lies between 0.01 and 1.0 mμg per ml. The good agreement with the photometric determination shown by the visual method of measuring growth response justifies continued use of the latter procedure for the nutritional classification of soil bacteria involving large numbers of isolates, in the grouping of which importance should not be attached to small growth differences brought out by the more refined procedure.

The ability of soil organisms with simple nutritional requirements to synthesize substances capable of promoting growth of bacteria requiring soil extract (or vitamin B_{12}) was examined by preparing culture filtrates from 20 strains by the procedure previously indicated. The effect of these (0.5 ml filtrate added to 5.0 ml medium Y) on promoting growth of the 30 test organisms is summarized in table 2. The ability of the test bacteria to grow is plainly related to the B_{12}

TABLE 2

Replacement of soil extract by culture filtrates of different vitamin B_{12} content as growth factor for 30 test organisms

CULTURE FILTRATE NO.	VITAMIN B_{12} (MμG/ML)		TEST ORGANISMS SHOWING GROWTH	DEGREE OF TURBIDITY						AVERAGE TURBIDITY SCORE
	In culture filtrate	In test medium		4	3	2	1	tr.	0	
SS 112	152.5	13.9	30	27	3	0	0	0	0	3.90
SB 54	62.0	5.6	30	24	5	1	0	0	0	3.77
B 3	57.1	5.2	30	1	16	12	1	0	0	2.57
I 2	42.5	3.9	30	25	2	1	2	0	0	3.67
MS 3	28.2	2.6	30	25	4	1	0	0	0	3.80
B 187	20.5	1.9	30	22	7	1	0	0	0	3.70
SS 131	10.0	0.9	29	20	5	3	1	0	1	3.40
W 42	6.7	0.6	30	16	12	2	0	0	0	3.47
B 88	4.2	0.38	30	13	12	4	1	0	0	3.23
Ma 35	3.3	0.30	30	15	11	2	2	0	0	3.30
B 6	1.5	0.14	29	5	9	9	6	0	1	2.37
B 81	0.88	0.08	30	5	5	14	6	0	0	2.30
B 253	0.77	0.07	1	0	0	0	0	1	29	0.02
B 35	0.62	0.06	1	0	0	0	0	1	29	0.02
B 21	0.48	0.04	0	0	0	0	0	0	30	0
B 7	0.46	0.04	0	0	0	0	0	0	30	0
B 89	0.21	0.02	0	0	0	0	0	0	30	0
B 11	0.17	0.015	0	0	0	0	0	0	30	0
B 52	0.17	0.015	0	0	0	0	0	0	30	0
B 225	0.10	0.01	0	0	0	0	0	0	30	0
Controls										
Medium Y			0	0	0	0	0	0	30	0
Medium Y + culture medium			0	0	0	0	0	0	30	0
Medium YS (0.49 mμg B_{12}/ml)			30	30	0	0	0	0	0	4.00
Medium YB_{12} (2.0 mμg/ml)			30	24	6	0	0	0	0	3.80

values of the filtrates, the data pointing to a fairly sharply defined critical concentration of 0.07 to 0.08 mμg per ml, below which the metabolic solutions are unable to provide growth-promoting properties for the test organisms. Comparison of the results with those in table 1 shows that the metabolic fluids, in terms of their B_{12} content as assayed by the *L. leichmannii* method, do not promote growth at as low concentrations as does the pure vitamin. This is ascribed to the presence in the crude culture liquids of interfering or antibiotic substances sufficient to prevent growth at the more critical lower vitamin levels. At the higher

B_{12} levels where the concentration of vitamin is adequate, as in culture filtrate B3, such substances, though unable to prevent development of the test organisms, have apparently had the effect of preventing optimum growth normally occurring at the concentration of B_{12} supplied.

On the other hand, the soil extract used, on the basis of its vitamin B_{12} content (0.49 mμg per ml in medium YS), was somewhat more effective in promoting growth than either the pure vitamin or the culture filtrates at equivalent B_{12} levels. Whether this is to be ascribed to more adequate mineral supplements or to the presence of additional accessory growth-stimulating factors has not been determined.

DISCUSSION

The importance of vitamin B_{12}-active substances for the nutrition of a number of microorganisms has been recognized, following the discovery by Shorb (1947) of a factor (LLD), present in liver extract, required for growth of *Lactobacillus lactis* Dorner, and the demonstration that vitamin B_{12} is wholly or partially responsible for the LLD activity observed for liver extract (Shorb, 1948). Certain strains of other species of *Lactobacillus* have been found to require vitamin B_{12} under special cultural conditions, including *L. leichmannii* (Skeggs *et al.*, 1948; Hoffmann *et al.*, 1948) as well as strains of *Lactobacillus acidophilus*, *Lactobacillus delbrueckii*, and *Lactobacillus helveticus* (Kitay *et al.*, 1950). Mutant strains of *Escherichia coli* were found by Davis and Mingioli (1950) to require vitamin B_{12} or methionine, while Gall and Huhtanen (1951) have stated that two species of rumen bacteria require the vitamin. Furthermore, Hutner *et al.* (1949) have shown vitamin B_{12} to be essential for growth of the algal flagellate, *Euglena gracilis* var. *bacillaris*. Attention has been centered chiefly on a comparatively small number of organisms requiring vitamin B_{12}, most of them related taxonomically, special interest being devoted to the use of appropriate strains for microbiological assay procedures.

From the present studies, however, it is apparent that the requirement for vitamin B_{12} or its physiological equivalent is by no means restricted to small groups of organisms, but is shown by an important ecological group of bacteria comprising part of the indigenous microflora of the soil. Since soils of the type sampled give normally counts of 50 to 100 millions per gram, as determined by plating methods, it is estimated that 4 to 8 million bacteria per gram that depend upon growth-promoting substances in these soils may utilize vitamin B_{12} as an essential nutrilite. Such numbers will doubtless be subject to much variation, depending upon the soil type. Since the group of organisms depending upon soil extract is relatively less abundant in the rhizosphere of plants than in soil not affected by the root system, there are no grounds for attributing the occurrence of large numbers of bacteria requiring vitamin B_{12} to the excretion of this growth factor by the roots of growing plants, and the findings are thus consistent with the view that higher plants are not an important source of B_{12}.

The cultures requiring soil extract or vitamin B_{12} for growth have retained this characteristic for approximately one year after repeated transfer. However,

it was noted that certain strains requiring soil extract that did not respond to vitamin B_{12} when isolated acquired the ability to utilize this vitamin for growth after several months on artificial culture. Other strains remained unable to use B_{12}, thus being dependent upon other unidentified bacterial growth-promoting substances present in soil extract.

NOTE ON CLASSIFICATION

Although the taxonomy of the bacteria requiring vitamin B_{12} or its physiological equivalent is being made the subject of a special study, mention may be made of the types represented. For morphological and physiological observation, appropriate solid or liquid media may be employed by incorporation of soil extract or vitamin B_{12}. In the group studied, 14 types have been observed, including two gram-positive, two gram-variable, and one gram-negative cocci. However, the great majority of the organisms, comprising 70 per cent of the isolates, were found to be pleomorphic forms of which 8 distinct types have so far been observed. These organisms appear as medium to long, irregular rods in young culture, rapidly fragmenting as the culture ages to cocci or coccoid rods. With the exception of one gram-negative type all species exhibit much variability in staining reaction. A single species occurred as curved, unbranched, gram-negative filaments in young culture, with small gram-negative coccoids appearing in older culture. The forms included 6 chromogenic types. The majority of the organisms liquefied gelatin, reduced nitrates, and hydrolyzed starch though little or no acidity was formed from sugars.

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SUMMARY

For an important proportion of the indigenous bacteria of soil that depend upon essential growth factors supplied by soil extract, the growth-promoting effect of the latter could be replaced by vitamin B_{12}, though not by cobalt (Co^{++}). Various other soil bacteria having simple nutritional requirements were able to synthesize a growth-promoting factor, the nutrilite effect of which was related to the amount of vitamin B_{12} produced. A large proportion of the bacteria for which soil extract or vitamin B_{12} is essential consists of highly pleomorphic organisms.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS

XI. FURTHER OBSERVATIONS ON THE NUTRITIONAL CLASSIFICATION OF BACTERIA[1]

By I. L. Stevenson[2] and J. W. Rouatt[3]

Abstract

A review of the method developed in this laboratory in 1943 for the nutritional classification of soil bacteria has suggested slight amendments in certain differential media: (1) the substitution of vitamin-free casamino acids for a combination of amino acids, and (2) the addition of vitamin B_{12} to the growth factor media. In a comparative study with a newly proposed scheme of classification, the more selective plating medium advocated was found to be less suitable for the isolation of soil bacteria than the nonselective soil extract agar in the original method. Furthermore, the replacement of potassium nitrate with diammonium phosphate as source of inorganic nitrogen in the basal medium failed to cause any significant change in the nutritional grouping. Results from the nutritional classification of some 600 isolates by the two methods showed that the new procedure represents only a slight modification of the original system.

Introduction

The inadequacy of the classical biochemical tests for a rational grouping of the indigenous soil bacteria on a physiological basis has led to the development of a method of classification of these organisms on the basis of their nutritional requirements. Following a preliminary system based on the use of three differential media (13), Lochhead and Chase (6) proposed a classification procedure in which organisms isolated on a nonselective basis from soil extract agar were grouped according to growth response in seven media of different nutritional complexities. Seven main nutritional groups were recognized, ranging from organisms capable of maximum growth in a simple basal medium to types unable to develop with supplements of amino acids, growth factors, or yeast extract, but which require soil extract for growth.

Since its introduction, this system of nutritional classification has aided the study and understanding of the indigenous soil bacteria. The method has been used in studying the comparative nutritional requirements of rhizosphere and control soil flora (13), and has provided some useful information concerning the physiological activity of the root system of plants (7). The effect of season and manurial treatments (3) and the influence of various crop plants on the indigenous soil flora (12) have also been investigated using this technique. Furthermore, the method has been applied to advantage in the study of certain soil-borne plant diseases; a relationship between the relative incidence of certain nutritional groups and the intensity of the disease has been shown by Hildebrand and West for strawberry root rot, and by Rouatt and Atkinson (9) in the case of potato scab disease.

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Recently Taylor (10) has criticized several aspects of this procedure for nutritional classification with particular respect to the isolation medium, the inorganic nitrogen source, the formulation of the differential media and the incubation times. Taylor has proposed a new and 'simpler' scheme which he feels will distinguish five nutritional groups with somewhat greater accuracy.

This paper reports on a comparative study of the two nutritional schemes of classification undertaken to establish the validity of Taylor's suggested 'improvements'. At the same time, several amendments to the method of Lochhead and Chase (6) are introduced in view of new products appearing on the market since the inception of the method in 1943, and the further identification of growth factors present in soil extract (5, 8).

Experimental

Cultures for these nutritional studies were obtained by plating suitable dilutions of three field soils on soil extract agar without an additional energy source and on the isolation medium described by Taylor (10). The plates were incubated 14 days at 26° C. after which all colonies were picked from one or more of the plates. Colonies from plates of soil extract agar were subcultured in a soil extract semisolid medium and those picked from Taylor's isolation medium in a semisolid medium of the same composition. Approximately 100 colonies were picked for each soil from the two isolation media giving a total of some 600 organisms. Prior to final transfer to the nutritional media a five-day broth culture [medium *YS* (6)] of each organism was prepared. Each isolate was then transferred by loop inoculation to the seven differential media described by Lochhead and Chase (6); to the same differential media with diammonium phosphate substituted for potassium nitrate as the inorganic nitrogen source; and to the five differential media of Taylor (10).

Two modifications of the differential media of Lochhead and Chase (6) have been introduced since this scheme was first reported. The substitution of 0.4% vitamin-free casamino acids (Difco) in place of the 10 amino acids formerly used in the preparation of medium *A* and medium *AG*, and the addition of 2 μgm. per liter of vitamin B_{12} to medium *G* and medium *AG*.

After incubation of the differential media for five days at 26° C. the organisms were classified nutritionally according to growth response. Each organism was grouped according to the original system (6) in which only maximum growth is considered significant and also by Taylor's system (10) in which any growth at all is considered sufficient for assignment to a nutritional group.

Results

Comparison of Isolation Media

One of the major differences in the nutritional scheme described by Taylor (10) is the use of a more selective medium than the soil extract agar proposed by this laboratory. Soil extract agar was chosen as being the least selective medium and designed to favor the indigenous soil types. Furthermore, colonies

developing on this medium after a relatively long incubation period are small, a circumstance considered favorable in that antagonistic and stimulative effects are kept at a minimum. Taylor's medium contains, in addition to soil extract and salts, yeast extract and tryptone, which he considers necessary for the development of some organisms requiring vitamins and other nutrients found in too small quantities in soil extract alone.

A study of the two isolation media was carried out and comparisons were made on the basis of: (i) the suitability as plating media for the enumeration and isolation of the soil flora; and (ii) the suitability of the media as a source of the indigenous soil flora.

A total of 18 soils were plated on the two media and in 15 cases a higher number of organisms was obtained on the soil extract agar. On the average, these numbers were approximately 30% higher than those of Taylor's medium after 14 days' incubation at 26° C. Records of numbers of five soils at 5, 8, and 14 days' incubation are given in Table I. The incubation time used by Taylor (10) was not specified; it is possible that the higher counts reported by him on his medium were due to too short a period. It is evident from our data that counts were generally higher on Taylor's isolation medium at five days, after which numbers on soil extract agar gradually increased as the more slowly developing types appeared.

A major consideration in the choice of a medium for the isolation of organisms from a mixed population is that antagonism between colonies be kept at a minimum, and that the surface of the agar be kept relatively free from spreading organisms. Because of the richness of its composition and resulting less selective nature, Taylor's isolation medium quickly gave rise to numerous zymogenic soil types whose growth habits often obscured the surface of the plates in as little as 24-48 hr. Similarly, this medium produced much larger colonies than the small 'distinct' colonies obtained by the use of soil extract agar. Fig. 1 illustrates typical plates of the two isolation media compared in these studies.

TABLE I

EFFECT OF INCUBATION TIME ON NUMBERS OF BACTERIA APPEARING ON SOIL EXTRACT AGAR AND TAYLOR'S ISOLATION MEDIUM

Soil	Isolation media					
	Soil extract agar			Taylor's isolation medium		
	Incubation time, days					
	5	8	14	5	8	14
N	133.0[1]	153.0	162.7	135.0	135.6	137.8
X_1	24.4	83.1	156.9	57.1	132.0	170.6
B. C.	28.0	61.0	75.0	36.0	39.0	47.0
B_1	25.6	41.8	61.6	27.4	31.6	34.4
X_2	49.0	92.0	141.2	70.6	85.4	92.8

[1]*Bacteria per gram oven-dried soil.*

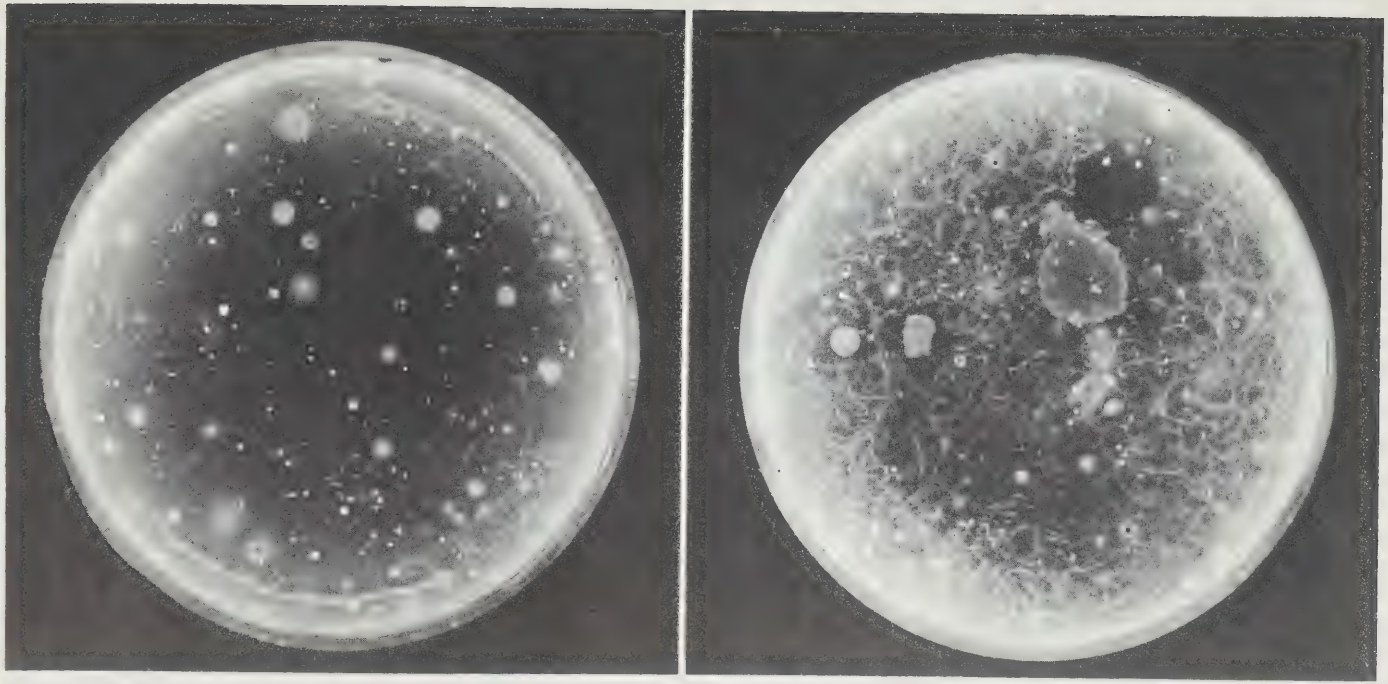

FIG. 1. Surface view of plates of soil extract agar and Taylor's isolation medium after 14 days' incubation at 26° C. Left—soil extract agar; right—Taylor's isolation medium.

Further proof of the unsuitable characteristics of Taylor's medium was obtained on the calculation of the Indices of Dispersion (1) for each set of replicate plates. Results of sets of Taylor's plates showed an extremely high percentage of abnormal variance while distribution of the soil extract series fell within the normal limits. It is apparent that the antagonistic processes between organisms appearing on Taylor's medium seriously affects the chance distribution of organisms on the plates, with the result that on isolating organisms from such plates a true 'cross-section' of the soil flora is not obtained.

During the course of this experiment, a total of 295 organisms were picked from soil extract agar and 285 from Taylor's isolation medium in order to make a nutritional comparison of the isolates from the two media. Each organism was inoculated into the seven nutritional media described by Lochhead and Chase in 1943 and classified according to tubes showing maximum turbidity at the end of incubation. Results of the classification are given in Table II. An analysis was performed on the data to test the homogeneity of the two bacterial populations. A nonsignificant χ^2 value was obtained showing a nutritional uniformity between the group of organisms isolated from Taylor's medium and the group isolated from soil extract agar. The fact that soil extract agar is capable of supporting a population nutritionally equivalent to that of Taylor's medium minimizes the importance of the stress placed by Taylor on the enrichment of the isolation medium to accommodate those organisms supposedly requiring more nutrients than provided in soil extract.

The Differential Media

Some objection has been raised (10) to the precipitation of calcium and magnesium during the preparation of our basal medium. To minimize this, it has been a constant practice to adjust to pH 6.8 before heating and allowing to cool before filtration. The basal medium thus prepared is found to obtain an adequate supply of calcium and magnesium and is completely free from precipitates which might be confused with light growths. In a comparative

TABLE II

A NUTRITIONAL COMPARISON OF ISOLATES FROM SOIL EXTRACT AGAR AND TAYLOR'S ISOLATION MEDIUM

Isolation media	Nutritional groups[1]						
	I	II	III	IV	V	VI	VII
Soil extract agar (Lochhead and Chase)	4.4	8.1	3.1	5.8	34.6	3.4	40.6
Taylor's isolation medium	5.6	11.2	2.5	6.7	37.5	5.6	30.9

[1] *Percentage of total organisms.*

test encompassing some 600 soil isolates, 122 organisms showed visible growth in the basal medium of Lochhead and Chase while only 98 responded to the calcium-free basal medium described by Taylor. It is difficult to understand the omission of calcium by Taylor who has previously reported on the growth stimulating properties of this essential mineral (11).

The possibility of the ammonium ion being a more suitable source of inorganic nitrogen than the nitrate used in our basal medium has also been suggested in view of the reported failure of some soil organisms to assimilate nitrogen as nitrate. To check the preference of soil organisms for their source of inorganic nitrogen, 583 isolates were inoculated into two series of the seven nutritional media of Lochhead and Chase. One series contained potassium nitrate and the other diammonium phosphate as the inorganic nitrogen source. Results of this survey are presented in Table III.

Statistical analysis of the data shows no variation in the nutritional grouping due to the nitrogen source. It appears that that portion of the soil flora with which we are concerned will utilize either nitrate or ammonia equally well as the inorganic nitrogen source.

The substitution of vitamin-free casamino acids is suggested for the amino acid mixture formerly used in the preparation of the medium containing the more complex forms of nitrogen. Apart from the advantage in ease of preparation, this medium supported good growth of many organisms that grew poorly in the synthetic amino acid medium. Of some 224 organisms requiring amino acid mixture, 193 showed equal or better growth in medium prepared with 0.4% vitamin-free casamino acids.

The proposed addition of vitamin B_{12} to our growth factor medium (medium *G*) results from the studies of Lochhead and Thexton (8) who have reported that a large proportion of soil organisms formerly believed to require soil extract were stimulated equally well in the presence of this vitamin. Comparative studies have shown a higher percentage of organisms appearing in the growth factor medium with vitamin B_{12} present than in its absence. As more and more unknown growth factors are identified in soil extract these will be placed in a medium of known composition so that eventually it is hoped that a well-defined medium can be prepared for even the most exacting soil micro-organisms.

TABLE III

EFFECT OF INORGANIC NITROGEN SOURCE ON THE NUTRITIONAL CLASSIFICATION OF SOIL BACTERIA

Nitrogen source in basal medium	Nutritional groups[1]						
	I	II	III	IV	V	VI	VII
KNO_3	5.0	9.6	2.8	6.2	36.0	4.5	35.9
$(NH_4)_2HPO_4$	3.7	10.1	3.4	5.8	37.0	6.8	33.2

[1] *Percentage of total organisms.*

Comparative Studies of Nutritional Groupings

In his recent criticism of the method used in this laboratory for classifying soil organisms, Taylor proposes a 'simpler' method of grouping utilizing five differential media. Unfortunately, Taylor provides no data permitting a comparison of the results of both methods.

Before a valid comparison of the two schemes of classification can be undertaken certain essential differences of the methods should be clarified. One major difference in the two schemes is the method of assigning the organisms to the nutritional groups. In our procedure, tubes containing differential media are incubated five days at 26° C., and then read for growth response. A value of 4 is given to the tube showing heaviest growth and the others rated by comparison. Readings of 1 and 2 are regarded as showing submaximal growth and are not considered significant. Lochhead (4) has recently stressed the importance of this method of evaluation. Taylor (10) gives no description of his method of assessing growth but it is assumed that any growth at all is considered significant.

Differences in the composition of the differential media also tend to make comparisons difficult. There is, however, a great deal of similarity between the nutritional media in both classifications. Both schemes employ a basal mineral salts medium. Likewise both use a medium to which amino acids are added in addition to the mineral salts (Lochhead's medium *A* and Taylor's medium II). It will be noted that Taylor's scheme provides no equivalent for the growth factor medium of Lochhead and Chase. Whether it is necessary to retain our medium *G* is a matter of personal preference. It has been found of value in studying the changes in microbiological equilibrium in

TABLE IV

A COMPARISON OF METHODS OF GROWTH EVALUATION AND THEIR EFFECT ON THE NUTRITIONAL GROUPING OF SOIL BACTERIA

Method of growth evaluation	Nutritional groups[1]						
	I	II	III	IV	V	VI	VII
Classification of Lochhead and Chase							
Lochhead and Chase	5.0	9.6	2.8	6.2	36.0	4.4	36.0
Taylor	20.7	13.5	4.6	10.0	32.7	8.0	10.5
Classification of Taylor							
Lochhead and Chase	0.9	7.5	9.5	55.7	26.4	—	—
Taylor	17.2	17.0	11.2	35.0	19.6	—	—

[1] *Percentage of total organisms.*

TABLE V

A COMPARISON OF THE CANADIAN AND ENGLISH SYSTEMS OF NUTRITIONAL CLASSIFICATION ON THE BASIS OF ANY GROWTH BEING SIGNIFICANT

Classification		Media composition						
		Mineral salts	Amino acids	Growth factors	Amino acids + growth factors	Yeast extract	Soil extract	Yeast and soil extracts
Lochhead and Chase (1943)	Medium	*B*	*A*	*G*	*AG*	*Y*	*S*	*YS*
	% organisms	20.7[1]	13.5	4.6	10.0	32.7	8.0	10.5
				14.6			18.5	
Taylor (1951)	Medium	I	II	—	III	IV plus tryptone	—	V plus tryptone
	% organisms	17.2	17.0		11.2	35.0		19.6
				11.2			19.6	

[1] *Percentage of total organisms.*

soil (2, 9); however it can be omitted in view of the presence of medium *AG*. Again, there is a similarity between the amino acid – growth factor media; medium III of Taylor and medium *AG* of our classification.

In a comparative study, approximately 600 soil isolates were classified nutritionally by the method described by the Canadian workers and also by Taylor's method. From the data shown in Table IV, it is evident that there is no similarity between the classifications obtained when both of the previously described methods of assigning nutritional groups are used. In both schemes of classification, where any growth at all is considered significant a much higher percentage of organisms naturally falls into the basal medium group. Where submaximal growth is not considered, many such organisms are not grouped until significant growth is obtained in one of the more complex media.

A true assessment of the value of either method of nutritional classification can only be made if growth response in the differential medium be determined in an identical manner. Extremely heavy growth and the formation of pellicles in the tryptone-containing media IV and V of Taylor restricts the use of our method in the assigning of many organisms to their nutritional groups. Table V presents the comparative classifications of a group of 594 soil isolates using Taylor's method of assigning the organisms to their nutritional groups. As has been previously mentioned there is a great deal of similarity between the compositions of media *B*, *A*, *AG* to media I, II, and III respectively. It is not surprising therefore that only an extremely slight variation appears in the percentage of organisms in these groups. The totalling of organisms appearing in our groups III and IV is permissible in view of the omission of a growth factor medium in Taylor's classification. Organisms of groups VI and VII may also be totalled for comparison with Taylor's group V organisms. Observation of the groups of organisms requiring the more complex growth requirements again shows only a slight difference in numbers. Taylor describes the organisms of group IV as being dependent on factors present in tryptone and that the yeast extract is probably unnecessary. From the data of Table V, it appears that tryptone has little or no influence on the organisms appearing in this group, since an approximately equal number of organisms exhibit growth in the presence of yeast extract alone (medium *Y*). Comparison of the percentage of organisms requiring growth factors present in soil extract in the two classification procedures shows little difference and again no notable change can be attributed to tryptone. It is evident from these data that the organisms appearing in groups IV and V are dependent on the yeast and soil extracts and that the presence of tryptone is unnecessary.

Discussion

The Canadian system (6) of classifying soil bacteria on the basis of nutritional needs has been found to differ in a number of respects from the scheme recently proposed (10). The suggestion that the soil extract agar does not contain sufficient concentrations of carbon and nitrogen for the development

of many organisms has not been borne out in these studies. It has been shown that the majority of soils plated exhibit higher numbers on soil extract agar than on the rich and selective plating medium described by Taylor. Although analysis has shown that nutritionally the populations of the two isolation media are relatively uniform, the spreading growth of certain spore-forming, zymogenic bacteria and the associated antagonistic effects renders Taylor's proposed isolation medium much less suitable than a nonselective substrate.

Certain criticisms (10) concerning the composition of the differential media of Lochhead and Chase (6) have been found to be unjustified. Results have shown that a higher proportion of soil isolates are capable of growth in our basal medium than in the calcium-free basal salts medium described by Taylor (10). The substitution of diammonium phosphate for potassium nitrate as the inorganic nitrogen source has failed to bring about any significant shift in the nutritional groupings of soil isolates. It is evident that either ammonia or nitrate serves as a suitable source of inorganic nitrogen. The replacement of the amino acids originally prescribed for media *A* and *AG* with 0.4% vitamin-free casamino acids is suggested in view of the improved growth of many organisms in an amino acid medium prepared from this product. Since the recognition of the importance of vitamin B_{12} to a large group of soil bacteria (8) this substance has been added to the growth factors present in media *G* and *AG*.

Contrary to the results of Taylor many organisms have been found dependent on factors present in yeast extract that are not supplied in the amino acid – growth factor medium. The value of tryptone in the new scheme of nutritional grouping has been exaggerated by Taylor who has allowed it to obscure the importance of yeast and soil extracts to many soil bacteria. Comparative studies have shown that numbers of organisms developing on medium *Y* (yeast extract) are approximately equal in number to those developing on Taylor's medium IV (yeast extract, tryptone). Similarly, numbers developing on media *Y*, *YS* (yeast–soil extracts) were equivalent to those developing on Taylor's medium V (yeast–soil extracts, tryptone). From these results it can be concluded that factors in yeast and soil extract are responsible for the growth of organisms in Taylor's media IV and V and that the presence of tryptone, as far as it affects the nutritional grouping, is superfluous.

Increase of incubation time from 5 to 12 days was found to have no effect on the nutritional grouping of the soil isolates. It is felt that the five-day period originally described is sufficient to obtain an accurate estimate of growth response.

A comparison of the two schemes of nutritional classification has shown the grouping of organisms by both methods is basically the same. In view of the ineffectiveness of tryptone it appears that the 'improved' scheme reported by Taylor is nothing more or less than a slight modification of the earlier proposed system of Lochhead and Chase. The only major difference is the method of

assessing growth and the assignment of organisms to their nutritional groups. In our opinion the difference between slight growth and heavy growth is far more significant in a scheme of nutritional classification than that between no growth and a trace of growth.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS

XII. CHARACTERISTICS OF VITAMIN-B_{12}-REQUIRING BACTERIA[1]

By A. G. Lochhead and Margaret O. Burton

Abstract

Ten types of bacteria isolated from soil are described for which vitamin B_{12} acts as an essential nutrilite. In addition to vitamin B_{12}, thiamine was found to be essential for the growth of all, and biotin for eight, of the 10 forms; in two cases riboflavin acted as a growth antagonist. For nine of the organisms thymidine, with or without the addition of purine and pyrimidine bases, was unable to replace vitamin B_{12} in promoting growth; with one organism but slight response was noted. Methionine was equally ineffective as a substitute for B_{12}. Three of the organisms were observed to be cocci and one a pleomorphic, curved, unbranched, filamentous form producing cocci in older culture. The remaining six types, representing approximately 80% of the isolates studied, were considered to belong to the genus *Arthrobacter*, the findings providing further evidence of the importance of this group as members of the indigenous soil microflora.

Introduction

Earlier studies of the nutritional requirements of the indigenous soil bacteria (9, 10) have indicated the presence of a group of organisms dependent for maximum growth upon a factor or factors present in aqueous extracts of soil. Some of these bacteria show no growth, others at best slight growth, in otherwise adequate media containing inorganic salts and sugars, supplemented by yeast extract or combinations of amino acids and vitamins (not including B_{12}). More recently it was shown (11, 12) that for an important proportion of the bacteria for which soil extract was essential for development the growth promoting effect of the latter could be replaced by vitamin B_{12}. It was further observed (12) that other soil bacteria, with simple nutritional needs, were able to synthesize a factor whose nutrilite effect on the organisms responding to the vitamin was related to the amount of B_{12}-active substance produced. The B_{12}-requiring organisms were estimated to constitute 7.6% of the bacteria capable of being isolated by non-selective plating procedures from the soil studied. From the findings reported it was apparent that the requirement for vitamin B_{12} or its physiological equivalent is by no means restricted to small groups of organisms, e.g. lactobacilli, but is shown by an important ecological group of bacteria forming part of the indigenous microflora of the soil.

The previous work (12) was based largely on a study of 30 cultures isolated from soil by plating on soil extract agar. Following the nutritional grouping of 534 isolates by the method of Lochhead and Chase (10), they were found to show no growth in yeast extract medium but to be capable of maximum growth upon the addition of soil extract or of crystalline vitamin B_{12}. The present report deals with the physiological and morphological characteristics of the group and suggests taxonomic relationships.

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Experimental

Since their isolation four years previously, stock cultures had been maintained in soil extract semisolid medium (Med. *SESS*). Upon retesting after this interval it was found that of the 30 strains which showed no growth in the yeast extract medium (Med. *Y*) seven strains had acquired the ability to develop in the absence of B_{12} while four showed slight growth. Consequently the studies here reported were confined to 19 cultures that continued to show no growth in the absence of B_{12} though developed well with the addition of the vitamin or of soil extract (Med. *YB*$_{12}$, *YS*). The media referred to were prepared as follows:

Soil extract semisolid (*Med. SESS*).—Soil extract, 1 liter (prepared by autoclaving 1 kgm. field soil with 1 liter tap water for 30 min. at 15 lb., filtering after the addition of a little calcium sulphate, and making filtrate up to 1 liter); K_2HPO_4, 0.2 gm.; yeast extract (Difco), 1.0 gm.; agar, 3 gm.

Yeast extract medium (*Med. Y*).—Glucose, 1.0 gm.; potassium monohydrogen phosphate, 1.0 gm.; potassium nitrate, 0.5 gm.; $MgSO_4 \cdot 7H_2O$, 0.2 gm.; calcium chloride, 0.1 gm.; sodium chloride, 0.1 gm.; $FeCl_3 \cdot 6H_2O$, 0.01 gm.; distilled water, 1 liter (basal medium); yeast extract, 1.0 gm.

Yeast + soil extract medium (*Med. YS*).—Basal salts glucose medium, 750 ml.; soil extract, prepared as above, 250 ml.; yeast extract, 1.0 gm.

Yeast + vitamin B$_{12}$ *medium* (*Med. YB*$_{12}$).—Medium *Y*, as above, plus crystalline vitamin B_{12} to give a concentration of 2 mμgm. per ml.

The morphological and physiological properties of the organisms were studied by employing appropriate solid or liquid media in which soil extract or vitamin B_{12} (2 mμgm. per ml.) was incorporated. In the absence of soil extract it was found important to include, along with B_{12}, an additional vitamin source such as yeast extract (0.1%) in order to provide other essential growth factors or, in later experiments, the specific additional vitamins found to be required.

Morphological and Physiological Types

On the basis of comparative tests of their morphology, general physiology, and vitamin requirements the organisms could be classified into 10 different types, the chief characteristics being summarized in Tables I to III. Six of the 10 types (I to VI), representing approximately 80% of the strains, were found to show the pleomorphism characteristic of the soil corynebacteria in that they change from rod forms of the 'diphtheroid' type in young culture to coccoid forms in older culture. They are considered to be representative of the genus *Arthrobacter* proposed by Conn and Dimmick (3) for the inclusion of soil types which, though related to *Corynebacterium*, were considered to have characteristics sufficiently different from those of the type species to warrant generic distinction. The members of this group showed variability in Gram

TABLE I

MORPHOLOGICAL AND PHYSIOLOGICAL CHARACTERISTICS

Type No.	Morphology	Endospores	Gram stain	Motility	Pigmentation (agar)	Gelatin liquefaction	Potato	Milk	Glucose	Sucrose	Lactose	Starch hydrolysis	Nitrate reduction	Indole	Catalase	Urease	Utilize nitrates as sole source of N other than vitamins	Utilize citrate as sole source of C other than vitamins	Growth at 37° C.
I	Pleomorphic—rods, coccoids	−	±	−	−	+	No. gr.	Coag.	Acid	−	−	+	+	−	+	−	+	+	−
II	Pleomorphic—rods, coccoids	−	±	−	−	+	No. gr.	No. ch.	−	−	−	+	+	−	+	Sl.	+	+	−
III	Pleomorphic—rods, coccoids	−	±	−	Pale brown	+	No. gr.	No. ch.	−	−	−	−	+	−	+	−	−	−	−
IV	Pleomorphic—rods, coccoids	−	±	−	Brown	+	No gr.	No. ch.	Acid	Acid	−	+	+	−	+	Sl.	+	−	−
V	Pleomorphic—rods, coccoids	−	−	−	Salmon pink	−	No. gr.	No. ch.	−	−	−	+	+	−	+	−	−	−	−
VI	Pleomorphic—rods, coccoids	−	±	−	−	+	No. gr.	Coag.	−	Alk.	−	−	−	−	+	−	−	−	−
VII	Pleomorphic—filaments, rods, coccoids	−	−	−	Yellow	+	No. gr.	Digest	−	Alk.	−	Sl.	+	−	+	−	−	−	+
VIII	Coccus	−	±	−	−	+	No. gr.	Coag.	Acid	Acid	Acid	+	+	−	+	−	−	−	+
IX	Coccus	−	±	−	−	+	Orange growth	Viscid	Acid	Acid	Alk.	−	+	−	+	−	−	−	+
X	Coccus	−	±	−	−	+	No. gr.	Coag.	Acid	−	−	+	+	−	+	−	+	+	+

staining, were non-motile, showed no growth at 37° C., and, in the majority of cases, liquefied gelatin, hydrolyzed starch, and reduced nitrates; acid production from sugars, if any, was not pronounced.

Types VIII to X showed, in all cases observed, round to coccoid cells and formed a group of bacteria which, though varying in other respects, liquefied gelatin, reduced nitrates, and grew well at 37° C. The remaining type, No. VII, exhibited striking morphological features. Young cultures consist of thin, flexuous, unbranched, Gram-negative filaments of varying length, including long, curved elements up to approximately 50 μ long. In somewhat older cultures the longer filaments appear to break up into shorter portions which, preparatory to division, swell at both ends to oval or round shape assuming a dumbbell form. The connection becomes thinner and finally disappears leaving oval or coccoid cells. The impression is thus one of pulling apart rather than of separation by breaking away. In certain preparations the swelling of the filaments may produce spindle shapes. After several days, cultures usually consist of a mixture of curved filaments and coccoid forms while in old cultures the latter predominate. At all stages of growth the cells are uniformly Gram-negative. Motility has not been observed. Benton (1) has described two types of chitinovorous bacteria, later referred to as myxobacteria (2), which exhibit certain morphological characters resembling those of Type VII. However, the identity of our type with this group has not been confirmed and its taxonomic status is still in doubt.

Vitamin Requirements

In the selection of media suited to the growth of the B_{12}-requiring organisms it was noted that in addition to vitamin B_{12} some source of other essential growth factors was required, such as yeast extract or soil extract. In order to determine more specifically the supplementary vitamin requirements of the group, experiments were carried out as summarized in Table II. A basal medium was prepared consisting of the basal salts as outlined above (cf. Med. *Y*); glucose, 1.0 gm.; casamino acids, 1.0 gm.; and vitamin B_{12}, 2 μgm. per liter. The vitamins employed were added in the following amounts per 100 ml. of medium: biotin, 0.1 μgm.; thiamine, 50 μgm.; calcium pantothenate, 50 μgm.; pyridoxine, 50 μgm.; pyridoxal, 50 μgm.; pyridoxamine, 10 μgm.; niacin, 50 μgm.; riboflavin, 50 μgm.; inositol, 5 mgm.; choline, 2 mgm.; *p*-aminobenzoic acid, 50 μgm.; and folic acid, 2 μgm. Inoculation was made from soil extract semisolid agar and cultures incubated at 26° C. To eliminate any carry-over effect, transfers were made from all cultures showing growth into tubes of similar medium and this serial transfer was repeated at least three times. A similar procedure was followed in Series 2 to note the effect of biotin and thiamine which vitamins had been found to be significant from the findings of Series 1. Tests were made in duplicate.

The results clearly indicated the importance of biotin and thiamine for the growth of the vitamin-B_{12}-requiring organisms, thiamine being essential for all, and biotin for eight of the 10 types. Of special interest is the role of

TABLE II

VITAMIN REQUIREMENTS IN ADDITION TO B_{12}

Type No.	Series 1: Effect of omitting single vitamins from combination (Basal medium = salts, glucose, casamino acids, vitamin B_{12})											Series 2: Effect of biotin and thiamine with B_{12} (salts, glucose, casamino acids)						Essential vitamins other than B_{12}
	Control (all)	−Biotin	−Thiamine	−Pantothenic acid	−Pyridoxine, pyridoxal, pyridoxamine	−Niacin	−Riboflavin	−Inositol	−Choline	−PAB	−Folic acid	B_{12}	Biotin thiamine	B_{12} biotin	B_{12} thiamine	B_{12} biotin thiamine	B_{12} all vitamins	
I	+	−	−	+	+	+	+	+	+	+	+	−	−	Tr.	−	+	+	Biotin, thiamine
II	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	Thiamine
III	+	−	−	+	+	+	+	+	+	+	+	−	−	Tr.	−	+	+	Biotin, thiamine
IV	+	Tr.	Tr.	+	+	+	+	+	+	+	+	−	−	Sl.	−	+	+	Biotin, thiamine
V	+	−	−	+	+	+	+	+	+	+	+	−	−	Tr.	−	+	+	Biotin, thiamine
VI	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	Biotin, thiamine
VII	−	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	Biotin, thiamine
VIII	+	+	−	+	+	+	+	+	+	+	+	−	−	−	+	+	+	Thiamine
IX	+	−	−	+	+	+	+	+	+	+	+	−	−	−	−	+	+	Biotin, thiamine
X	+	Tr.	−	+	+	+	+	+	+	+	+	−	−	−	Sl.	+	+	Biotin, thiamine

+, *good growth*; −, *no growth*; *Tr., trace of growth*; *Sl., slight growth.*

riboflavin in the nutrition of Types VI and VII. The suppression of growth in its presence indicates that this vitamin acts as an antagonist under the conditions prevailing in the semisynthetic medium used. Since these organisms grow in more complex media (*YB*$_{12}$, *YS*) which contain yeast extract and therefore riboflavin, this growth is ascribed to a reversal of inhibition by substances not present in the simpler medium.

It is known that certain bacteria responding to vitamin B_{12} and employed in assay procedures for this vitamin do not react specifically to B_{12} but show responses to certain physiologically related compounds. That thymidine as well as other desoxyribosides may replace vitamin B_{12} in the nutrition of lactobacilli, e.g. *Lactobacillus leichmannii*, has been shown by various investigators (14, 15, 16). Certain B_{12}-requiring mutants of *Escherichia coli* have been isolated by Davis and Mingioli (4) which, in contrast to the behavior of many lactic acid bacteria, do not respond to thymidine; however, methionine serves as an alternate nutrilite for B_{12}. The use of the algal flagellate *Euglena gracilis* var. *bacillaris* for the assay of B_{12} was proposed by Hutner *et al.* (6) who found thymidine to be inactive for this organism. In various hands it has shown much promise as an assay organism in view of the greater specificity of B_{12} for promoting growth, Robbins *et al.* (13) having shown methionine, among many compounds, to be inactive, although vitamin B_{12a} and B_{12b} were effective as substitutes for B_{12}.

TABLE III

COMPARISON OF VITAMIN B_{12}, METHIONINE, AND THYMIDINE

Type No.	Basal medium = salts, glucose, casamino acids, biotin, and thiamine			Plus adenine, guanine, xanthine, and uracil		
	B_{12}	Methionine	Thymidine	B_{12}	Methionine	Thymidine
I	+	−	−	+	−	−
II	+	−	−	Sl.	−	−
III	+	−	−	+	−	−
IV	+	−	−	+	−	−
V	+	−	−	+	−	−
VI	+	−	−	+	−	−
VII	+	−	−	+	−	−
VIII	+	−	−	+	−	−
IX	+	−	Tr.	+	Sl.	Sl.
X	+	−	−	+	−	−

+, *good growth;* −, *no growth; Tr., trace of growth; Sl., slight growth.*

Comparative tests were made of the effect of vitamin B_{12}, methionine, and thymidine on the 19 strains represented by the 10 types. Since it was shown (5) that thymidine effectively replaced B_{12} for certain strains of *Lactobacillus leichmannii* only in the presence of purine and pyrimidine bases (particularly a combination of adenine, guanine, and uracil), tests were made with and without the addition of adenine, guanine, xanthine, and uracil. A basal medium was used composed of salts, glucose, casamino acids, biotin, and thiamine at concentrations similar to those previously indicated. The test substances were employed as follows: vitamin B_{12}, 2 mμgm. per ml.; methionine, 20 μgm. per ml.; thymidine, 10 μgm. per ml.; and the purines and uracil each at 20 μgm. per ml. The methionine, in view of its presence in the casamino acids, represented an extra amount available.

The results, summarized in Table III, indicated that under the experimental conditions nine of the 10 bacterial types were unable to grow when B_{12} was replaced by thymidine or added methionine. One type, No. IX, representing one strain of the 19 under investigation, was able to show slight growth in the absence of B_{12}. Since it had been found to be the only strain able to grow on potato, which contains no measurable quantity of vitamin B_{12} by the rat assay procedure (7), this is ascribed to its ability to respond to compounds which are physiologically related to vitamin B_{12}.

Description of Types

Type I (*Cultures No. 7, 8, 10, 27, 56, 61, 110*)

In young cultures, rods varying in size and shape, with angular arrangement, 0.6–0.8 × 2–5 μ; curved, bent, and slightly swollen forms frequent with occasional rudimentary budding; Gram-variable; non-motile; becoming coccoid in older culture, cells 0.7–0.9 μ diameter, generally Gram-positive.

Agar colonies circular, 1–1.5 mm. diameter, convex, entire, glistening, cream colored; moderate growth on agar slant, filiform, slightly raised, smooth, glistening, butyrous, cream-colored; in gelatin stab, stratiform liquefaction, no surface growth, moderate sediment, liquid turbid; no growth on potato; milk (with added yeast extract and vitamin B_{12}) slowly coagulated; slight acidity from glucose, none from sucrose and lactose.

Starch hydrolyzed; nitrites produced from nitrates; indole not formed; catalase positive; urease negative; can utilize nitrate as nitrogen source and citrate as carbon source; requires biotin and thiamine in addition to vitamin B_{12}; aerobic; good growth from 20° to 32° C., slight growth at 10° C., no growth at 37° C.

Type II (*Cultures No. 17, 23, 28*)

In young cultures, rods, straight, curved or bent, generally 0.5 × 1.5–3.5 μ; snapping division giving angular arrangement of cells; most cells Gram-positive; non-motile; in older cultures cells coccoid to round, 0.6–0.8 μ, Gram-variable, mostly Gram-positive.

Agar colonies punctiform, gray-cream in color; agar slant cultures show slight to moderate growth, filiform, rather flat, smooth, waxy, butyrous, cream colored; in gelatin stab, slow stratiform liquefaction, no surface growth, slight sediment, liquid very turbid; no growth on potato; no change in milk after four weeks; no acidity from carbohydrates.

Starch hydrolyzed; nitrites formed from nitrates; indole not formed; catalase positive; slight production of urease; can utilize nitrates as nitrogen source and citrate as carbon source; requires thiamine in addition to vitamin B_{12}; aerobic; grows at 20° to 32° C. (best at 32° C.), trace of growth at 10° C., none at 37° C.

Type III (*Cultures No. 38, 100*)

Young cultures show irregular rods including straight, curved, bent, and sometimes swollen forms; mostly 0.5–0.6 × 1–3 μ with occasionally longer cells; snapping division; predominantly Gram-positive; non-motile; in older cultures coccoid to round cells, approximately 0.7 μ, mostly Gram-positive.

Agar colonies small, circular, up to 1.2 mm. diameter, gray, becoming pale brown in color; good growth on agar slant, filiform, slightly raised, smooth, glistening, butyrous; in gelatin stab culture, saccate liquefaction becoming stratiform, no surface growth, moderate sediment, liquid clear; no growth on potato; milk shows no change after four weeks; slight acidity from glucose, none from sucrose or lactose.

Starch not hydrolyzed; nitrates formed from nitrates; indole not produced; catalase positive; urease negative; can not utilize nitrate as sole source of nitrogen; requires biotin and thiamine in addition to vitamin B_{12}; aerobic; grows equally well at temperatures between 20° and 32° C., only a trace of growth at 10° C., no growth at 37° C.

Type IV (*Culture No. 40*)

In young cultures, rods of varying size, mostly 0.5–0.6 × 1–3.5 μ; cells may be curved or slightly swollen; snapping division giving angular arrangement; mostly Gram-positive, non-motile; in older cultures cells coccoid, 0.8 μ in diameter, Gram-positive to Gram-variable.

Agar colonies, circular, up to 2 mm. diameter, convex, entire, glistening, brown in color; abundant growth on agar slant, filiform, slightly raised, smooth, glistening, butyrous, light brown in color; in gelatin stab, stratiform liquefaction, brown surface growth, moderate sediment, slight turbidity; no growth on potato; no change in milk after four weeks; slight acidity from glucose and sucrose.

Starch hydrolyzed; nitrites formed from nitrates; indole not produced; catalase positive; slight production of urease; can utilize nitrate as nitrogen source; unable to utilize citrate as sole source of carbon with nitrate as sole source of nitrogen; requires biotin and thiamine in addition to vitamin B_{12}; aerobic; grows well between 20° and 32° C., trace of growth at 10° C., no growth at 37° C.

Type V (*Culture No. 62*)

In young cultures, rods, straight or curved, mostly 0.4–0.6 × 1–3 μ; snapping division giving angular arrangement; Gram-negative, non-motile; in older cultures Gram-negative cocci, 0.7–0.8 μ in diameter.

Agar colonies small, circular, 0.8 mm. diameter, convex, glistening, pink; agar slants show moderate growth, filiform, slightly raised, surface somewhat rough, waxy luster, soft cheesy consistency, salmon pink in color; gelatin stab culture shows salmon colored surface growth but no liquefaction; no growth on potato; no change in milk; no acidity from glucose, sucrose, or lactose.

Starch hydrolyzed; nitrites produced from nitrates; indole not formed; catalase positive; urease negative; can not utilize nitrates as sole source of nitrogen; requires biotin and thiamine in addition to vitamin B_{12}; aerobic; grows from 20° to 32° C., maximum growth at 32° C., no growth at 10° C. or 37° C.

Type VI (*Culture No. 76*)

In young cultures, short to medium rods, straight to slightly curved, with snapping division, mostly 0.6 × 0.8–2 μ; Gram-positive to Gram-variable; non-motile; in older cultures, short rods, coccoid and round cells, 0.7–0.8 × 0.7–1.0 μ, mostly Gram-positive.

Agar colonies punctiform; agar slant cultures, moderate growth, filiform, slightly raised with irregular surface, glistening, butyrous, cream colored; in gelatin stab, saccate liquefaction, becoming stratiform, no surface growth, abundant sediment, liquid clear; no growth on potato; milk slowly coagulated; no acidity from glucose, sucrose, or lactose.

Starch not hydrolyzed; nitrates not reduced to nitrites; indole not produced; catalase positive; urease negative; unable to utilize nitrates as sole source of nitrogen; requires biotin and thiamine in addition to vitamin B_{12}; in semi-synthetic medium riboflavin acts as growth antagonist; aerobic; grows well between 20° C. and 32° C. (best at 32° C.), no growth at 10° C. or 37° C.

Type VII (*Culture No. 30*)

In young culture, rods of varying length and width extending to curved, unbranched, non-motile filaments attaining a length of 40–50 μ, width, 0.4–0.7 μ; as culture ages filaments break up into shorter portions forming a chain of rods which later become dispersed; coccoids appear later by process involving swelling of ends of rods to produce dumbbell appearance, the connecting portion becoming thinner and finally disappearing, leaving coccoid or round cells, approximately 0.8 μ; in older cultures coccoids usually somewhat smaller, 0.6–0.7 μ; at all stages of growth, Gram-negative.

Agar colonies punctiform; agar slant cultures, moderate growth, filiform, flat, smooth, glistening, soft butyrous, yellowish to yellowish-brown in color; gelatin stab, stratiform liquefaction, no surface growth, moderate yellowish

sediment, liquid clear; no growth on potato; milk cultures, slow but eventually strong digestion, liquid yellowish, reaction acid; no acidity from glucose, lactose, slight alkalinity in sucrose medium.

Weak diastatic action on starch; nitrites produced from nitrates; indole not formed; catalase positive; urease negative; unable to utilize nitrate as sole source of nitrogen; requires biotin and thiamin in addition to vitamin B_{12}; in semisynthetic medium riboflavin acts as growth antagonist; aerobic; grows at 20° C. to 37° C. (best at 32° to 37°), no growth at 10° or 45° C.

Type VIII (*Culture No. 12*)

Cocci, occurring singly, in pairs, short chains, and groups; 0.7–0.8 μ in diameter; Gram-negative (in some preparations a minority of the cells may be Gram-positive); non-motile.

Agar colonies circular, up to 1.8 mm. in diameter, convex, entire, opaque, glistening, gray-white; agar slant cultures, moderate growth, filiform, slightly raised, smooth, glistening, butyrous, cream colored; in gelatin stab, stratiform liquefaction, no surface growth, slight sediment, liquid turbid; no growth on potato; milk slowly coagulated with some clear liquid near top, pale brownish color, reaction acid; growth enhanced in presence of carbohydrates, slight acidity from glucose, sucrose, and lactose.

Starch hydrolyzed; nitrites formed from nitrates; indole not produced; catalase positive; urease negative; unable to utilize nitrate as sole source of nitrogen; requires thiamine in addition to vitamin B_{12}; aerobic; grows well between 20° and 37° C., slight growth at 10°, no growth at 45° C.

Type IX (*Culture No. 54*)

Cocci, occurring singly, in pairs, short chains, and groups; 0.6–0.8 μ in diameter; Gram-variable; non-motile.

Agar colonies punctiform; agar slant cultures, moderate growth, filiform, surface irregular, slightly glistening, membranous consistency, cream colored; in gelatin stab, stratiform liquefaction, slight surface growth, slight sediment, liquid faintly turbid, viscid; on potato, slight, filiform, orange-colored growth, becoming rough and dry; no change in milk after four weeks, older cultures show some sediment and gray brownish discoloration and become viscid; growth enhanced, with acid production in presence of glucose and sucrose, not with lactose.

No diastatic action on starch; nitrites formed from nitrates; indole not produced; catalase positive; urease negative; unable to utilize nitrate as sole source of nitrogen; requires biotin and thiamine in addition to vitamin B_{12}; in absence of B_{12}, slight growth with methionine and thymidine; aerobic; grows between 20° and 37° C. (best at 32° to 37°), no growth at 10° or 45° C.

Type X (*Culture No. 86*)

Cocci, occurring singly, in pairs, short chains, and groups; 0.8–1.0 μ in diameter; Gram-variable; non-motile.

Agar colonies circular, up to 1 mm. in diameter, convex, entire, glistening, gray-cream colored; on agar slant growth moderate to abundant, filiform, slightly raised, surface smooth and glistening, cream colored; in gelatin stab, saccate liquefaction becoming stratiform, no surface growth, slight sediment, liquid turbid; no growth on potato; milk coagulated with slightly pink coloration, reaction acid; growth enhanced in presence of sugars, slight acidity from glucose.

Starch hydrolyzed; nitrites produced from nitrates; indole not formed; catalase positive; urease negative; able to utilize nitrate as source of nitrogen and citrate as sole carbon source; requires biotin and thiamine in addition to vitamin B_{12}; aerobic; grows well between 20° and 37° C. (best at 32°), slight growth at 10° C., no growth at 45° C.

Observations

Previous reports (9, 10) had drawn attention to the occurrence in soil of considerable numbers of B_{12}-requiring bacteria, estimated at approximately four to eight million per gram in the soil under investigation. Vitamin B_{12} is known to occur in soil, its presence being attributable to the decomposition of organic matter of animal origin, as well as to its synthesis by soil microorganisms, many of which have been shown to possess this ability. That many forms are dependent upon the vitamin, therefore, is not surprising.

Results of the present study suggest that many types of soil bacteria responding to B_{12} show a high degree of specificity in their requirement for this vitamin as an essential nutrilite. That this appeared to be the case with nine of the 10 types described is believed to be due to the highly selective procedure employed in the nutritional differentiation of the cultures following their isolation from soil. This involved the separation, as a distinct group, of organisms responding to a factor or factors occurring in soil extract but not present in yeast extract.

That six of the 10 types of bacteria described were considered to be members of the genus *Arthrobacter* is further evidence supporting the belief that the 'soil diphtheroids' constitute an important proportion of the indigenous microflora of the soil. Though the type species, *Arthrobacter globiforme*, has relatively simple nutritional requirements, being able to develop with inorganic nitrogen and requiring no added accessory growth substances (3), other species which show close taxonomic relationship by morphological criteria have specific vitamin demands and include the forms described in the present study as well as others with highly specific growth factor requirements (8). That such fastidious organisms have not been generally recognized is believed to be ascribed to the use of inappropriate media for the enumeration, isolation, and study of the soil bacteria many of which are dependent upon one or more of the many growth factors present in soil extract.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS: XIII. EFFECT OF DECOMPOSITION OF VARIOUS CROP PLANTS ON THE NUTRITIONAL GROUPS OF SOIL BACTERIA

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Although the microbiological equilibrium of the soil is subject to certain variations through the influences of such factors as season, temperature, moisture, and fertilizer treatment, it appears to undergo its greatest alteration through the effect of the growing plant, which causes striking changes, both quantitative and qualitative, in that portion of the soil coming within its influence, the rhizosphere. The influence of the living plant is manifest, not only in a marked increase in total numbers of microorganisms in soil adjacent to the root system, but in characteristic shifts in the balance between different bacterial groups, whether classified on the basis of morphology, physiological activity, or nutritional requirements.

The incorporation of plant material in soils, in the form of either crop residues or green manures, is known likewise to exert an effect on the microbial population of the soil. In general, addition of plant material results in an initial rise in the numbers of microorganisms, the rapidity and extent of which are dependent upon the age and nature of the material as well as the character of the soil. Later there is usually a more gradual decrease in numbers, which continues as the decomposition progresses and the soil reverts to its more stabilized condition.

The extensive investigations carried out by Waksman, Tenney, Starkey, and their various associates in New Jersey have greatly elucidated the process of organic matter decomposition and have provided a sound basis for our knowledge of the mechanisms involved in the transformation by microbial action of different plant materials added to soil and of the process of humus formation. Other workers, including Martin, Jensen, Vandecaveye, Norman, Smith, Kalnins, Bodily, Bartholomew, Broadbent, Bremner, Dawson, Lehner, and their associates have contributed to our knowledge of the changes involved as organic matter, added in the form of plant residues, is subjected to decomposition processes by soil microorganisms under a variety of environmental conditions.

In addition to studying the chemical changes accompanying the breakdown of plant material, various workers have observed the coincident changes in the micropopulation through determinations of total numbers of bacteria, actinomycetes, or fungi or of the abundance of physiological groups, such as cellulose-decomposing forms or bacteria concerned with nitrogen transformation.

The object of the present investigation was to study changes in the microbiological balance, with respect to the relative incidence of different nutritional

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groups of bacteria, after plant material had been allowed to decompose in soil, and to compare the effects of different crop plants.

MATERIAL AND METHODS

Three series of greenhouse experiments were conducted in successive years on different field soils. Six crops; namely, wheat, oats, flax, timothy, alfalfa, and red clover, were grown in the soils in 9-inch pots, five replicates per crop. Uncropped series were maintained as controls. Approximately midway through the growth period the crops were removed from the soil, the plants from all pots of the same crop combined and chopped up, and 200 g. of each was incorporated with the soil from which the plants had been removed. The soil was then replaced in the five pots. The pots were maintained at a constant weight by addition of water at frequent intervals.

At two stages of decomposition (table 1) composite samples of soil were prepared from each series of pots, and suitable dilutions were plated on soil extract agar without added energy material. This medium was chosen as being the least selective for soil bacteria. It was prepared by autoclaving 1 kg. field soil with 1 liter tap water for 30 minutes at 15 pounds, filtering after addition of a little $CaSO_4$, and making the filtrate up to 1 liter. To the filtrate were added 0.02 per cent K_2HPO_4 and 1.5 per cent agar, with final pH of 6.8. Plates were incubated at 26°C. for 14 days. After the plates were counted, all colonies were picked from suitable plates or sectors (approximately 100 for each sample), and stab inoculations were made into soil extract semisolid (containing 0.02 per cent K_2HPO_4, 0.1 per cent yeast extract, 0.3 per cent agar) to serve as stock cultures for further study.

The organisms isolated were differentiated according to nutritional requirements by the method of grouping previously described by Lochhead and Chase (6), whereby growth response is determined in a series of media of increasing complexity, ranging from a simple basal medium of inorganic salts and sugar to a

TABLE 1

Plan of experiments on decomposition and rhizosphere effects of six crop plants

Year	Decomposition Studies		Rhizosphere Studies	
	Time following incorporation in soil at time of analysis	Number of cultures classified	Age of plants at time of analysis	No. of cultures classified
	days		*weeks*	
1952	31	769	—	—
	59	751		
1953	42	701	6	1,222
	70	710		
1954	28	711	6	1,238
	56	705		
	Total number of cultures classified	4,347	Total number of cultures classified	2,460

complex medium containing both yeast extract and soil extract. The following six media were used with certain modifications as indicated:

Basal medium (*medium B*).
Amino acid medium (*medium A*)—vitamin-free casamino acids (0.4 per cent) used in place of the original mixture of amino acids.
Amino acid + growth factor medium (*medium AG*). Medium A plus growth factors (6), which did not include vitamin B_{12}.
Yeast extract medium (*medium Y*).
Yeast extract + B_{12} medium (*medium* YB_{12}). Medium Y plus vitamin B_{12} (2 μg. per liter), a new medium introduced to differentiate bacteria requiring this vitamin (7).
Yeast + soil extract medium (*medium YS*).

The differential media (4 ml. in 13- by 100-mm. test tubes) were inoculated by loop transfer (1 mm.) from a 5-day culture of each isolate in medium YS. After 5 days' incubation at 26° the growth responses were read and the organisms assigned to their respective groups according to the method previously described (6).

In addition to the investigation of the decomposition effects of the six crops, studies were made during the last 2 years of the rhizosphere effects of the same crop plants (table 1). For these tests, samples of rhizosphere and control soils were taken when the crops were removed. To obtain the rhizosphere samples, several plants, the number depending on the crop and representative of all pots of the same crop, were carefully removed from the soil. The roots were shaken to remove loosely adherent soil and placed in measured amounts of sterile water in weighed flasks, sufficient roots being added to provide enough soil to approximate the 1:100 dilution of the control soil samples. These control soil samples consisted of composite specimens of soil remaining in the pots after removal of the plants. The roots were removed after appropriate dilutions were plated, and the contents of the flasks, as well as the control soil suspensions were evaporated to dryness to allow estimation of the plate counts on a dry-weight basis, with correction for aliquots removed. The procedure for plating and for the nutritional differentiation of the bacteria was similar to that already described.

RESULTS

Table 2 summarizes the results of separate tests made in 3 years on the effect of the six cover crops, incorporated in soil and sampled at two stages of decomposition, on the relative incidences of the nutritional groups of bacteria. The findings are based on the examination of 4,347 bacterial isolates. Table 3 presents, for comparison, results of studies of the rhizosphere effects of the same crop plants while growing in the respective soils during the second and third years of the experiments. This work involved the nutritional grouping of 2,460 cultures.

The data from the rhizosphere studies (table 3) not only indicate a pronounced increase in total numbers of bacteria, but further emphasize certain characteristic effects of plant growth on the bacterial microflora of the soil immediately adjacent to the root system, to which attention has been directed in previous reports from this laboratory. Chief of these effects is the preferential stimulation, in the

TABLE 2

Effect of incorporation in soil of six cover crops on percentage incidences of nutritional groups of bacteria

Year	Time of Decomposition	Crop	Plate Count	Number of Isolates Tested	Incidence of Nutritional Groups					
					B	A	AG	Y	YB_{12}	YS
	days		*millions/g.*		%	%	%	%	%	%
1952	31	Wheat	77	106	17.0	15.1	16.9	45.3	2.9	2.8
		Oats	81	112	25.0	25.9	14.4	31.2	2.7	0.8
		Flax	108	114	13.2	16.7	18.4	46.5	2.6	2.6
		Timothy	82	106	10.4	10.4	17.9	58.5	2.8	0.0
		Alfalfa	84	114	1.7	13.1	14.1	58.8	11.4	0.9
		Red clover	106	104	5.8	7.7	11.5	69.2	5.8	0.0
		Control	77	113	5.3	7.1	8.9	67.2	7.1	4.4
	59	Wheat	80	119	11.8	17.6	19.3	48.8	0.0	2.5
		Oats	54	112	28.8	23.4	13.6	28.8	1.8	3.6
		Flax	65	108	18.5	17.6	15.7	46.3	1.9	0.0
		Timothy	60	104	11.5	3.9	15.4	64.4	2.9	1.9
		Alfalfa	70	108	10.2	6.5	8.3	62.0	3.7	9.2
		Red clover	59	94	8.5	20.2	12.7	54.3	1.1	3.2
		Control	41	106	6.6	15.1	10.4	60.3	1.9	5.7
1953	42	Wheat	132	93	0.0	21.5	11.8	50.6	12.9	3.2
		Oats	184	102	8.8	21.6	17.7	27.4	11.7	12.8
		Flax	200	95	4.2	12.7	9.4	51.6	12.7	9.4
		Timothy	210	103	2.9	19.4	23.3	41.8	9.7	2.9
		Alfalfa	160	100	4.0	22.0	2.0	29.0	30.0	13.0
		Red clover	180	105	3.8	14.3	4.7	53.4	16.2	7.6
		Control	110	103	4.9	6.7	3.8	62.2	8.8	13.6
	70	Wheat	110	101	3.9	20.9	8.9	54.5	7.9	3.9
		Oats	92	93	11.8	27.9	10.8	40.9	6.5	2.1
		Flax	210	102	6.8	16.7	7.8	57.9	9.9	0.9
		Timothy	160	102	1.9	23.6	16.7	48.0	3.9	5.9
		Alfalfa	148	104	3.8	19.3	9.7	50.9	15.4	0.9
		Red clover	117	103	4.8	13.6	5.8	63.2	9.7	2.9
		Control	80	105	1.9	11.5	8.5	60.9	5.7	11.5
1954	28	Wheat	219	103	6.7	18.5	8.7	46.7	8.7	10.7
		Oats	184	107	2.8	26.2	11.2	51.4	4.7	3.7
		Flax	104	91	10.9	19.8	4.5	51.7	2.2	10.9
		Timothy	302	101	16.8	13.9	9.9	36.7	15.8	6.9
		Alfalfa	244	99	4.0	26.3	16.1	27.3	24.3	2.0
		Red clover	368	102	8.8	19.6	10.8	46.1	13.7	1.0
		Control	48	108	5.6	12.9	3.7	62.1	2.8	12.9
	56	Wheat	180	102	3.9	20.6	6.9	58.9	7.8	1.9
		Oats	158	97	4.1	16.4	5.2	68.1	2.1	4.1
		Flax	102	94	7.4	19.1	1.1	61.7	4.3	6.4
		Timothy	210	102	10.8	19.6	7.8	49.1	10.8	1.9
		Alfalfa	221	104	9.7	23.2	4.8	48.2	19.3	3.8
		Red clover	180	100	8.0	11.0	7.0	57.0	14.0	3.0
		Control	54	106	9.4	6.6	4.7	66.1	3.8	9.4

TABLE 3

Rhizosphere effect of six cover crops on percentage incidences of nutritional groups of bacteria

Year	Crop		Plate Count	Number of Isolates Tested	Incidence of Nutritional Groups					
					B	A	AG	Y	YB_{12}	YS
			millions/g.		%	%	%	%	%	%
1953	Wheat	C*	84	88	1.2	19.2	3.4	47.7	12.6	15.9
		R	640	107	3.7	62.7	9.3	12.1	0.9	11.2
	Oats	C	97	99	0.0	22.2	29.3	40.4	6.0	2.1
		R	500	107	6.5	61.8	12.1	9.4	1.8	8.4
	Flax	C	65	101	3.9	10.9	15.9	45.6	8.9	14.8
		R	420	106	8.5	35.8	21.7	23.7	2.8	7.5
	Timothy	C	102	102	5.9	16.7	9.8	46.1	8.8	12.7
		R	1100	106	5.7	59.4	5.7	14.2	7.5	7.5
	Alfalfa	C	87	99	1.0	19.2	14.1	38.1	14.4	13.2
		R	940	106	8.5	43.4	17.0	13.2	13.2	4.7
	Red clover	C	88	96	6.2	11.5	13.5	48.9	13.6	6.3
		R	890	105	4.8	40.0	14.3	33.3	2.8	4.8
1954	Wheat	C	112	111	6.3	17.1	7.2	52.2	8.1	9.0
		R	690	105	8.6	52.4	6.7	18.1	2.9	11.1
	Oats	C	92	99	3.0	16.1	12.1	49.5	6.1	13.2
		R	790	102	5.9	43.1	4.0	29.4	2.0	15.6
	Flax	C	87	105	13.3	7.6	9.5	57.2	3.8	8.6
		R	520	110	9.1	52.8	15.5	13.6	4.5	4.5
	Timothy	C	134	100	2.0	22.0	11.0	45.0	12.0	8.0
		R	1480	109	7.4	44.1	11.0	18.3	11.0	8.2
	Alfalfa	C	108	93	0.0	12.9	9.6	62.4	7.5	7.5
		R	990	99	6.0	50.5	4.0	21.3	12.2	6.0
	Red clover	C	118	105	7.6	15.2	1.9	48.6	9.5	17.1
		R	1120	99	10.1	38.4	4.0	31.3	8.1	8.1

* C = control; R = rhizosphere.

rhizosphere, of bacteria requiring amino acids for maximum growth (group A). Noted also is a generally lowered incidence of bacteria requiring for maximum growth the more complex substances in yeast and soil extract. This is particularly noted with organisms of group Y, responding to yeast extract. These shifts in the microbial equilibrium are found for all six crops studied, and the findings (other than those for alfalfa with respect to vitamin B_{12}-requiring bacteria, subsequently referred to) do not point to any specific crop differences.

The effects of incorporating plant material in soil, as examined at the times indicated, that is, within periods varying between 28 and 70 days, are less pronounced than the rhizosphere effects exerted by the same crop plants. As noted in table 2, total numbers of bacteria, as determined by plating, are slightly to moderately higher, after the times indicated, in the soils to which crop material was added than in the control soils. As various authors have previously noted (2, 4, 9, 10, 11, 12), however, there is normally a pronounced initial increase in numbers of bacteria upon the addition of plant material to soil and these num-

TABLE 4

Effect of incorporation of plant material on nutritional groups of bacteria
Averages of six crops

Year	Time of Decomposition	Incorporation	Incidence of Nutritional Groups					
			B	A	AG	Y	YB_{12}	YS
	days		%	%	%	%	%	%
1952	31	Crops	12.2	14.8	15.5	51.6	4.7	1.2
		Control	5.3	7.1	8.9	67.2	7.1	4.4
	59	Crops	14.9	14.9	14.2	50.8	1.9	3.4
		Control	6.6	15.1	10.4	60.3	1.9	5.7
1953	42	Crops	4.0	18.6	11.5	42.3	15.5	8.2
		Control	4.9	6.7	3.8	62.2	8.8	13.6
	70	Crops	5.5	20.3	10.0	52.6	8.9	2.8
		Control	1.9	11.5	8.5	60.9	5.7	11.5
1954	28	Crops	8.3	20.7	10.4	43.3	11.6	5.9
		Control	5.6	12.9	3.7	62.1	2.8	12.9
	56	Crops	7.3	19.4	5.5	57.2	9.7	3.5
		Control	9.4	6.6	4.7	66.1	3.8	13.2
Grand average of all crops.....			8.7	18.1	11.2	49.6	8.7	4.2
Grand average of all controls.....			5.6	10.0	6.7	63.1	5.0	10.2

bers may reach a maximum within a very few days (2). This is followed by a decrease, more gradual than the initial rise, as conditions slowly approach a more stabilized state. The present work showed no significant differences in numbers of bacteria suggestive of a specific crop effect, a finding supporting results of Dawson (4), who found no differences in total counts that could be attributed to the kind of residue used.

With regard to the effect of the incorporated plant materials on the balance between the various nutritional groups of soil bacteria, the results (table 2) indicate a much less pronounced influence than that exerted by the same crop plants while growing in the soil. But the data do point to shifts in the nutritional grouping of the organisms similar in kind to, though less in degree than, the rhizosphere effect, in that there is a trend toward higher proportions of organisms with the simpler requirements (groups B, A, and AG), and to lower relative incidences of bacteria requiring yeast or soil extract (groups Y, YS). This is illustrated in table 4, showing average percentages for all six crops. The percentages of the special group YB_{12} are less characteristic, the averages for the crops being raised by the higher incidences of B_{12}-requiring forms in the case of alfalfa. Apart from this instance, the findings suggest no specific effects considered attributable to the kind of crop material used.

ALFALFA AND VITAMIN B_{12}

The only suggestion of a specific, as distinct from a general, crop effect brought out by the data concerns the relationship of alfalfa to vitamin B_{12}. From the

effect. Appropriate dilutions were plated on plain soil extract agar without added energy material, chosen as being the least selective medium for the indigenous soil bacteria. It was prepared by autoclaving 1 kg. of field soil with 1 liter of tap water for 20 minutes at 15 lb., filtering, and bringing the volume to 1 liter. To the filtrate were added 0.02% K_2HPO_4 and 1.5% agar, with final pH = 6.8. After 14 days' incubation at 26° the plates were counted and from suitable plates or sectors all colonies were picked (approximately 100 for each replicate) and stab inoculations made into soil extract semisolid (soil extract plus 0.02% K_2HPO_4, 0.1% yeast extract, 0.3% agar) to serve as stock cultures for further study. In all, 499 cultures were so isolated from the five replicate samples and considered as a group for determination of their vitamin requirements.

Vitamin Requirements

The basal medium consisted of inorganic salts, glucose, and vitamin-free 'Casamino acids'. It was prepared by adding to 1 liter of distilled water: K_2HPO_4, 1.0 g.; KNO_3, 0.5 g. ; $MgSO_4.7H_2O$, 0.2 g.; $CaCl_2$, 0.1 g.; NaCl, 0.1 g.; $FeCl_3.6H_2O$, 0.01 g. The solution was adjusted to pH 6.8, heated to boiling, cooled, and filtered; 1.0 g. glucose and 1.0 g. 'Casamino acids' were then added.

Determinations of the vitamin needs of the isolates were carried out in three stages. Each culture was first inoculated into the basal medium and into the basal medium plus all the vitamins. By this means all organisms capable of growing in the former, and thus not requiring vitamins, were eliminated. Those that responded to the complete vitamin mixture were submitted to a further test in which the vitamins were divided into two groups. The growth responses were noted upon the addition of each group, as well as of both groups, to the basal medium. In this way it was possible to eliminate, in many cases, a group of vitamins found to be non-essential. Depending upon the results of the group tests, the detailed requirements of the cultures were determined by preparing a series of appropriate media containing, respectively, the group or groups with essential factors and the same combinations minus each vitamin in turn. Basal medium controls were included in all test series.

The vitamins, with the amounts at which they were used per 100 ml. of medium, were as follows:

Group A.—thiamine, 50 μg.; calcium pantothenate, 50 μg.; biotin, 0.1 μg.; vitamin B_{12}, 0.2 μg.; folic acid, 10 μg.

Group B.—riboflavin, 50 μg.; pyridoxine 50 μg. (with pyridoxal, 50 μg.; and pyridoxamine, 10 μg.); *p*-aminobenzoic acid, 50 μg.; nicotinic acid, 50 μg.; choline, 2 mg.; inositol, 5 mg.

The various liquid media, dispensed in test-tubes, were inoculated by loop transfer (1 mm.) from the semisolid stock cultures. Growth responses were normally read after 5 days' incubation at 26° C. though a small percentage of more slowly growing organisms were kept longer. A vitamin was considered

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A relatively high proportion of the indigenous bacteria of a field soil (27.1%, corresponding to 14.1 millions/g.) required one or more vitamins for growth. The vitamins found to be essential, either alone or with others, were, in order of frequency, thiamine, biotin, vitamin B_{12}, pantothenic acid, folic acid, nicotinic acid, and riboflavin. In all, 16 different 'patterns' were noted for vitamin requirements, the number of vitamins needed by individual strains ranging from one to five. The findings point to the soil as an important habitat of vitamin-requiring bacteria, many of which show potentiality as assay organisms. Their occurrence in the numbers found indicates that growth-factors should receive equal emphasis with antibiotics in problems involving the microbial equilibrium in soil and interrelationships between the normal soil microflora, soil-borne disease organisms, and growing plants.

Introduction

Soil contains microorganisms of the most diversified characteristics, whether judged on the basis of morphology, physiological properties, nutritional characters, or synthetic abilities. Most of the B-vitamins, as well as other microbial growth factors, are known to be present in fertile soil (1, 5, 8, 9, 10). The presence of vitamins is attributable to release from vitamin-containing plant and animal residues, to liberation from the roots of growing plants, and, probably most important, to synthesis by microorganisms. It is therefore not surprising that bacteria are present for which certain vitamins are essential for growth.

It has been previously shown (6, 11) that among the indigenous soil bacteria are forms requiring one or more preformed vitamins. Subsequent reports (3, 4, 7) have shown the need of various types, more fully described, for certain specific factors, namely vitamin B_{12} and the terregens factor (TF). However, apart from a recent report (5) on the incidence of B_{12}- and TF- requiring bacteria in soil and in the rhizosphere of certain plants, no information is available concerning the abundance in soil of bacteria requiring specific vitamins for growth.

The purpose of the present study was to examine the specific B-vitamin requirements of the indigenous soil bacteria and to note the abundance of forms requiring the various growth factors.

Material and Methods

Source of Cultures

Five replicate samples of soil were taken from a field supporting a crop of barley, sufficiently distant from the plants to exclude any rhizosphere

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Contribution No. 421 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa.

results of the plant decomposition studies (table 2), as well as of the rhizosphere tests (table 3), the incidence of bacteria requiring vitamin B_{12} for maximum growth (group YB_{12}) was found, without exception, to be highest with alfalfa. Though solely on the basis of the experiments here reported this finding may be best stated as a trend, the results are supported by other observations made in this laboratory indicating that alfalfa bears a special relationship to vitamin B_{12}.

In a study of the capacity for synthesis of vitamin B_{12} by 70 strains representing six species of *Rhizobium*, Burton and Lochhead (3) found *Rhizobium meliloti* to be sharply distinguished from the other species by its ability to produce significantly higher quantities of the vitamin, one strain producing more than 1000 μg. per liter of culture fluid. Twelve strains of *R. meliloti* yielded an average of 410 μg., and 13 strains of *R. trifolii* an average of 29 μg., per liter. The capacity of these organisms to synthesize B_{12} has since been confirmed by Levin *et al.* (5). More recently, Lochhead and Burton (8), in describing 10 types of vitamin B_{12}-requiring bacteria isolated from soil, found nine of the types to be highly specific in their requirement for B_{12}. These organisms, for which desoxyribosides and methionine were quite ineffective as substitutes for crystalline B_{12}, were found to respond to culture filtrates of *R. meliloti* (unpublished findings), the results strengthening the evidence for the B_{12}-producing capacity of this organism.

The results of the present study, considered in light of other experimental work, support the hypothesis that the increased incidence of B_{12}-requiring organisms in the soil in which alfalfa was incorporated is due to a higher content of vitamin B_{12} than that in the soils to which the other crops were added. The plant material undergoing decomposition consisted of roots as well as top portions and thus, for the legumes, included nodular tissue. That the effect was pronounced with alfalfa and not with red clover is believed related to the B_{12} content of the respective nodules. In a separate experiment, analysis of alfalfa nodules for B_{12}-active substances gave a value of 454 mμg. per milligram dry weight, and that of red clover nodules, 16 mμg. The leafy portions of alfalfa are not believed to contribute significantly to the B_{12} content of the soil. Although earlier reports of the presence of this vitamin in alfalfa are conflicting, the application of newer assay techniques has shown that the apparent B_{12} activity of alfalfa (for example, alfalfa leaf meal) is to be attributed in large measure to other growth factors, such as naturally occurring desoxyribosides, which are active for certain less specific assay organisms (1).

SUMMARY

The effect of incorporating in soil six cover crops—wheat, oats, flax, timothy, alfalfa, and red clover—on the relative incidences of different nutritional groups of bacteria was studied. The "rhizosphere effects" of the same crop plants were studied for comparison.

Based on results of the nutritional differentiation of a total of 6,807 cultures, the findings indicated that incorporation of the plant materials exerted a much less pronounced effect on the balance between the nutritional groups than did

the same plants when growing. The data pointed to shifts similar in kind to, though less in degree than, the rhizosphere effect in showing a trend toward higher proportions of bacteria with the simpler requirements and lower relative incidences of organisms needing more complex nutrients.

Apart from the relationship of vitamin B_{12} to alfalfa, no effects were considered to be attributable to the kind of crop used. The incidence of vitamin B_{12}-requiring bacteria was found, in all cases, to be highest with alfalfa, in the rhizosphere as well as in soil following decomposition of plant material. The belief that such a relationship may exist is strengthened by the finding of much higher quantities of B_{12} in alfalfa nodules than in those of red clover, as well as by previous work in this laboratory which showed the alfalfa nodule organism, *Rhizobium meliloti*, to be capable of synthesizing significantly higher amounts of vitamin B_{12} than other species, including *R. trifolii*.

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Based on results of the nutritional differentiation of a total of 6,807 cultures, the findings indicated that incorporation of the plant materials exerted a much less pronounced effect on the balance between the nutritional groups than did

essential when no growth occurred on its omission from an otherwise adequate medium. In doubtful cases the tests were repeated and, if necessary, additional serial transfers made to eliminate any possible carryover effect.

Results

The main findings are summarized in Tables I and II. Of a total of 499 isolates, 135 or 27.1% required one or more vitamins for growth. On the basis of an average total bacterial count of 52.4 millions/g. for the five replicate samples examined it was estimated that one or more vitamins were essential for the development of 14.1 millions/g. of soil.

The vitamins found to be needed, either alone or with others, are shown in Table I in order of frequency, together with the estimated numbers of bacteria per gram of soil requiring them. With the majority of the strains (63%), more than one vitamin was required; only in the case of thiamine, biotin, and riboflavin were organisms found that needed but a single vitamin, representing, for the riboflavin group, all that gave response to this factor. Thiamine was the most frequently required vitamin with 19.2% of the total isolates finding it essential; the results thus provide further evidence of the need for this vitamin so frequently encountered. Biotin, as expected, proved to be likewise prominent among the growth factors required. However, of special interest was the finding of B_{12} as the third most commonly required vitamin, the results confirming previous studies (5, 7) that pointed to soil as an important habitat of vitamin B_{12}-requiring organisms.

For none of the isolated cultures was pyridoxine, *p*-aminobenzoic acid, choline, or inositol found to be essential though in some cases these factors stimulated growth. It is likely that bacteria requiring one or more of these factors occur in soil; however, the results suggest that their numbers, estimated in the present study at less than 100,000/gm., are relatively small.

TABLE I

VITAMIN REQUIREMENTS AND INCIDENCE OF BACTERIA RESPONDING TO VARIOUS GROWTH FACTORS

Vitamin required	Alone	With others	Total	% of total isolates	Approximate number/g. soil
Thiamine	26	71	97	19.4	10,200,000
Biotin	21	61	82	16.4	8,600,000
Vitamin B_{12}	0	36	36	7.2	3,800.000
Pantothenic acid	0	23	23	4.6	2,400,000
Folic acid	0	15	15	3.0	1,600,000
Nicotinic acid	0	10	10	2.0	1,000,000
Riboflavin	3	0	3	0.6	300,000
Pyridoxine	0	0	0	< 0.2	< 100,000
p-Aminobenzoic acid	0	0	0	< 0.2	< 100,000
Choline	0	0	0	< 0.2	< 100,000
Inositol	0	0	0	< 0.2	< 100,000

TABLE II

PATTERNS OF VITAMIN REQUIREMENTS

No. of cultures	Thiamine	Biotin	B_{12}	Pantothenic acid	Folic acid	Nicotinic acid	Ribo-flavin
26	+	−	−	−	−	−	−
21	+	+	−	−	−	−	−
23	+	−	+	−	−	−	−
2	+	−	−	+	−	−	−
11	+	+	+	−	−	−	−
1	+	+	−	+	−	+	−
5	+	+	−	−	−	+	−
5	+	+	−	+	+	−	−
1	+	+	−	+	−	+	−
2	+	+	−	+	+	+	−
21	−	+	−	−	−	−	−
2	−	+	+	−	−	−	−
2	−	+	−	+	−	−	−
8	−	+	−	+	+	−	−
2	−	+	−	+	−	+	−
3	−	−	−	−	−	−	+
Total 135	97	82	36	23	15	10	3

Requiring 1 vitamin	=	50 cultures
Requiring 2 vitamins	=	52 cultures
Requiring 3 vitamins	=	25 cultures
Requiring 4 vitamins	=	6 cultures
Requiring 5 vitamins	=	2 cultures
Total		135

Table II shows the various 'patterns' of vitamin requirements found, together with their frequency. In all, 16 'patterns' were recognized. For the great majority of the organisms, one or two or, less commonly, three vitamins were sufficient, though with small numbers four or even five were required for growth.

Observations on Selected Cultures

A number of cultures were selected for more detailed examination, chosen to include forms illustrative of the needs for the various seven vitamins, the effects of which were studied quantitatively. A basal salts–glucose–casamino acids medium, as described above, was used and vitamin additions made at concentrations previously indicated. The general procedure consisted in preparing duplicate 10-ml. quantities of each test medium in 50 ml. Erlenmeyer flasks which were inoculated with one drop of a washed suspension of the test organism which had been grown in a medium containing the complete vitamin mixture. The flasks were incubated in a reciprocal shaker at 26° C. for 2 days, or in some cases longer, depending on the culture, following which turbidity readings were made on a Klett–Summerson photometer, allowance being made for uninoculated controls. The series of media used were based on the results of the qualitative tests and thus varied with the culture, according to the vitamin or vitamins whose effects were being tested.

TABLE III

SELECTED CULTURES WITH DIFFERENT VITAMIN REQUIREMENTS—GROWTH RESPONSE IN DIFFERENTIAL MEDIA

Cult. no.	Vitamin(s) required	Average turbidity (Klett–Summerson)							
		Basal medium (no vit.)	11 vitamins	11 vit. minus riboflavin	Riboflavin alone	11 vit. minus thiamine	Thiamine alone	11 vit. minus biotin	Biotin alone
785	Riboflavin	6	317	12	312	—	—	—	—
824	Thiamine	47	206	—	—	43	233		
637	Biotin	3	262	—	—	—	—	6	290

		Basal medium (no. vit.)	11 vitamins	11 vit. minus nicotinic	Biotin, nicotinic, thiamine	Biotin, nicotinic, pantothenic
630	Biotin, nicotinic acid, thiamine	17	232	36	220	38
709	Biotin, nicotinic acid, pantothenic	11	110	28	14	115

		Basal medium (no. vit.)	Thiamine, biotin, pantothenic, folic, nicotinic	5 vit. minus thiamine	5 vit. minus pantothenic	5 vit. minus folic	5 vit. minus nicotinic
727	Thiamine, biotin, pantothenic, folic acid	2	167	19	1	17	144
619	Thiamine, biotin, pantothenic, folic acid, nicotinic acid	0	170	14	2	2	2

		Basal medium (no. vit.)	11 vitamins	11 vit. minus B_{12}	B_{12}, thiamine	B_{12}, biotin	B_{12}, thiamine, biotin	Factor B, thiamine, biotin
539	Vitamin B_{12}, thiamine (Factor B pos.)	16	285	13	289	25	302	284
541	Vitamin B_{12}, thiamine (Factor B neg.)	5	278	27	274	27	267	20
530	Vitamin B_{12}, thiamine biotin (stim.) (Factor B neg.)	13	283	19	160	25	272	15

The results are shown in Table III. The organisms needing but a single vitamin, No. 785, 824, and 637, show clear-cut requirements for riboflavin, thiamine, and biotin respectively. It is noted that vitamins other than the one in question were neither essential nor stimulatory, the single factor providing as good growth as the complete medium. Cultures No. 630 and 709 (which require biotin) are both seen to need nicotinic acid; however, they differ in that No. 630 requires, in addition, thiamine and No. 709 requires pantothenic acid. The table shows that for both No. 727 and 619, thiamine, pantothenic acid, and folic acid are essential (in addition to biotin) while No. 619 requires nicotinic acid as well.

TABLE IV

PLAN OF EXPERIMENTS ON VITAMIN RESPONSE BY SELECTED CULTURES

Culture No.	Vitamin	Range	Basal medium
785	Riboflavin	0.005–0.1 μg./ml.	Salts, glucose, Casamino acids
824	Thiamine	0.1 –5.0 mμg./ml.	Salts, glucose, Casamino acids
630	Nicotinic acid	0.005–0.1 μg./ml.	Salts, glucose, Casamino acids, thiamine, biotin
619	Pantothenic acid	0.05 –5.0 μg./ml.	Salts, glucose, Casamino acids, thiamine, biotin, folic acid, nicotinic acid
619	Folic acid	0.001–0.05 μg./ml.	Salts, glucose, Casamino acids, thiamine, biotin, pantothenic acid, nicotinic acid
541	Vitamin B_{12}	0.025–0.5 mμg./ml.	Salts, glucose, yeast extract (Difco)

Vitamin B_{12}, as well as thiamine, is required by cultures No. 539, 541, and 530, the latter being, in addition, stimulated by biotin, though this is not essential to growth. The table also illustrates differences with respect to the ability of Factor B,* the non-nucleotide portion of the molecule of vitamin B_{12}, to replace cyanocobalamin in the nutrition of certain strains. Whereas 539 is able to utilize Factor B, cultures No. 541 and 530 are unable to do so.

A number of the same cultures, having requirements for different vitamins, were studied in more detail with the object of noting their responses to varying concentrations of the vitamins in question. The general procedure was similar to that described above. The strains used, as well as the vitamins tested and the basal media employed, are indicated in Table IV. The composition of the media was based on previous findings, to include vitamins required by the organisms other than that being tested. In the case of culture No. 541, requiring B_{12}, yeast extract was used.

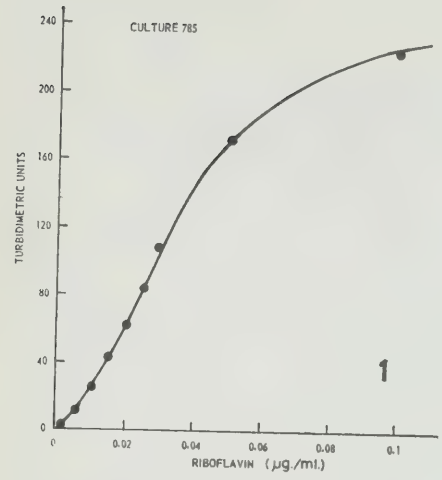
Following preliminary tests to find suitable concentration ranges, growth-response curves in respect to the six vitamins were established as shown in Figs. 1 to 6. Under the experimental conditions the essentiality of the vitamins for growth of the organisms in question was confirmed. The level at which response was evident varied with the factor; however, the effective concentration of a given vitamin might be expected to vary, depending upon the test organism selected.

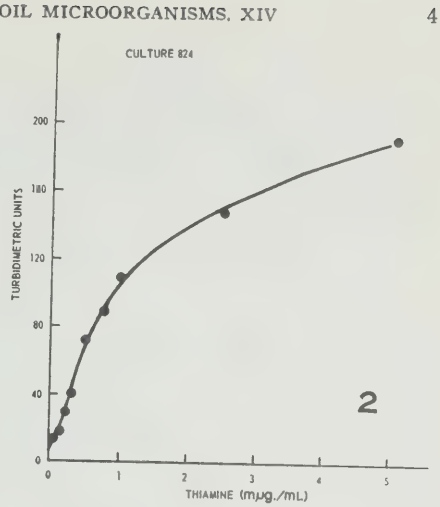
Discussion

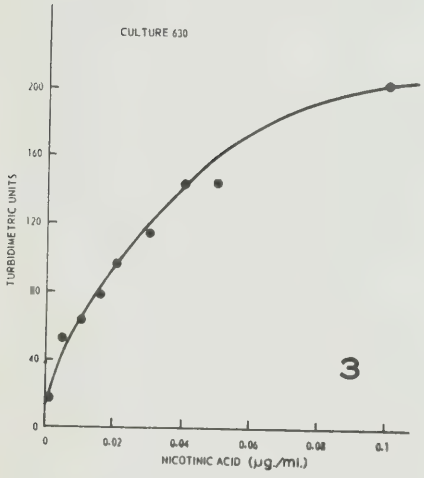
Microbial growth-promoting substances in soil and organisms requiring or synthesizing them have received less attention than the subject of antagonisms. Growth factors may be considered in the same category with antibiotics in so far as they affect the growth of many microorganisms at

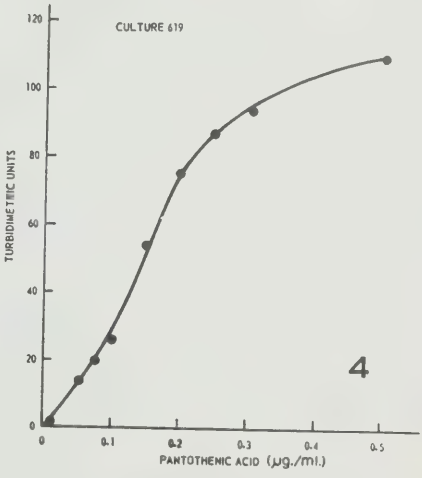
*Kindly supplied by Dr. S. H. Hutner from a sample obtained from Merck and Co., Inc., through courtesy of Dr. David Hendlin.

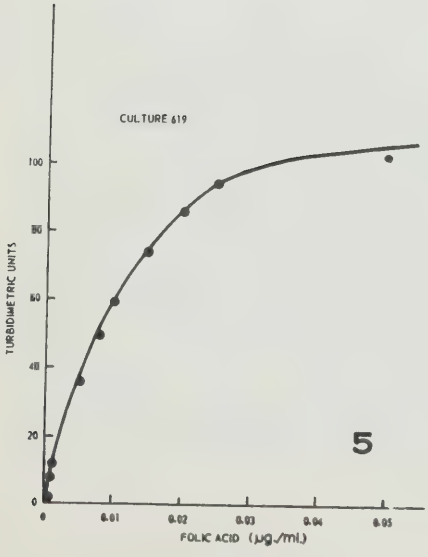
FIGS. 1–6. Response of cultures to six vitamins: Culture No. 785 to riboflavin; No. 824 to thiamine; No. 630 to nicotinic acid; No. 619 to pantothenic acid; No. 619 to folic acid; No. 541 to vitamin B_{12}.

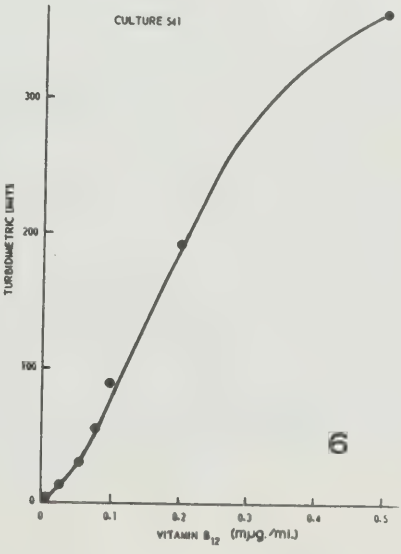

CULTURE 785
TURBIDIMETRIC UNITS
RIBOFLAVIN (µg./ml.)
1


CULTURE 824
TURBIDIMETRIC UNITS
THIAMINE (mµg./mL.)
2


CULTURE 630
TURBIDIMETRIC UNITS
NICOTINIC ACID (µg./ml.)
3


CULTURE 619
TURBIDIMETRIC UNITS
PANTOTHENIC ACID (µg./ml.)
4


CULTURE 619
TURBIDIMETRIC UNITS
FOLIC ACID (µg./ml.)
5


CULTURE 541
TURBIDIMETRIC UNITS
VITAMIN B12 (mµg./ml.)
6

very low concentrations and may be produced by microbial synthesis. Consequently growth-factor effects could well be as important as antibiotic activity in affecting the microbial equilibrium.

If the proportion of vitamin-requiring bacteria had been insignificant, their presence might be regarded as of minor importance; however, in view of their occurrence in the numbers found it is apparent that growth-promoting factors should be taken into account in any consideration of the microbial status or potentiality of soil. This appears to be particularly relevant to problems involving the complex interrelationships between the normal soil microflora, soil-borne plant disease organisms, and the plant itself. In such relationships, growth factors, which have been relatively ignored, could have a bearing as significant as that of antibiotics. Indeed it is possible that the lack of progress in applying antibiotic-producing organisms to the control of disease caused by soil-borne pathogens, as recently stressed by Garrett (2), may be attributed in some measure to neglect of the role of factors which promote, rather than suppress, the growth of many soil microorganisms.

The results also emphasize the wide diversity of the soil's indigenous microflora in its nutritional aspects and provide a further example of the interdependence of many of the soil organisms; thus those that require certain specific substances depend upon the synthetic activities of other forms that are less demanding in their nutritional needs. The findings are of some ecological significance in pointing to the soil as an important habitat of vitamin-requiring bacteria, many of them being, as far as known, characteristic of soil and representing hitherto undescribed species with potentiality as vitamin assay organisms.

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QUALITATIVE STUDIES OF SOIL MICROORGANISMS: XV. CAPABILITY OF THE PREDOMINANT BACTERIAL FLORA FOR SYNTHESIS OF VARIOUS GROWTH FACTORS

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It is now generally conceded that most, if not all, of the B-vitamins, as well as other microbial growth factors, are present in fertile soil (1, 18, 22, 23, 24, 26). Their occurrence has been ascribed to various processes—to release from vitamin-containing plant and animal residues, including organic fertilizers, to liberation from the roots of growing plants, and to synthesis by microorganisms. Although the relative importance of the various agencies is doubtless subject to change depending upon the nature of the soil, the rate of application of organic manures, and the extent of crop growth, the capacity of many soil microorganisms to synthesize vitamins appears to be an important factor in contributing to the vitamin supply of the soil. At the same time microorganisms are concerned with the destruction as well as with the production of vitamins, as pointed out by Starkey and Schmidt in studies of the levels of nicotinic acid, pantothenic acid, and riboflavin in decomposing organic materials (27) and in soil (25). Thus the concentration of a growth factor at any time depends upon the balance established between synthetic and destructive agencies.

Our knowledge of the direct relationship between soil microorganisms and growth factors is fragmentary, with particularly little information of a quantitative nature being available. Recently Lochhead and Burton (19, 20) in reporting on the incidence of bacteria having requirements for specific vitamins, found 27.1 per cent of 499 organisms, isolated from a field soil by nonselective procedures, to require one or more vitamins for growth. The number of vitamin-requiring forms was estimated at 14.1 millions per g. In all, 16 different "patterns" of vitamin requirements were noted, depending upon the varied needs of different strains. The same authors (18), in a study of 2877 isolates, compared the incidence of bacteria requiring vitamin B_{12} and the terregens factor (TF) in three control field soils and in the rhizospheres of three crop plants. The control soils were estimated to contain approximately 4 million bacteria per g. which required B_{12} for growth and roughly one-tenth as many for which TF was essential. Though the proportions of both groups were lower in the rhizospheres, their absolute numbers were much higher than in control soil in view of the general "rhizosphere effect."

Though it has been often stated that the production of vitamins by microorganisms represents an important source of supply of these substances in soil, little positive evidence was adduced before the studies of Schmidt and Starkey

[1] Contribution No. 436 from the Bacteriology Division, Science Service, Canada Department of Agriculture, Ottawa. The author is indebted to J. W. Rouatt for making cultures available and to Miss Agnes MacIntosh for her very efficient technical assistance.

(25), who determined the quantities of riboflavin and pantothenic acid present during the course of decomposition of plant residues incorporated into soil. Although in a natural soil, where different agencies act simultaneously in adding to and diminishing the vitamin supply, it is difficult if not impossible to evaluate each factor, these authors established that microorganisms may play a highly important role in the synthesis, as well as in the destruction, of vitamins.

While it has been shown (9, 13) that metabolic filtrates of certain soil bacteria not requiring vitamins may stimulate growth of other organisms dependent upon vitamins, there is little information on the capabilities of soil microorganisms for synthesis of specific growth factors, or to the numerical distribution of such forms in soil (to be sure, various vitamins have been shown to be produced by organisms isolated from soil). Thus in recent years interest in vitamin B_{12} has stimulated a search for organisms capable of synthesizing this vitamin, and various workers [cf. Darken (6)] have shown that many bacteria and actinomycetes, including strains isolated from soil, are able to produce B_{12}-active substances. Such studies have had little relationship to the microbial economy of soils as such. Lochhead and Thexton (14) showed, however, that B_{12}-synthesizing bacteria from soil were able to permit growth of B_{12}-requiring organisms isolated from the same soil in proportion to the amount of B_{12} produced. Synthesis by soil bacteria of the terregens factor found essential to the growth of fastidious organisms of a group typified by *Arthrobacter terregens* provides an analogous example of the dependence of soil organisms upon essential substances elaborated by others (15, 16).

No information appears to be available respecting the abundance in soil of microorganisms capable of producing vitamins or other essential growth factors. The studies here reported were undertaken to observe the incidence of bacteria able to synthesize various factors and to include a comparison of control and rhizosphere soils.

MATERIAL AND METHODS

Field samples were taken from a control soil and from the rhizospheres of rye and barley plants growing respectively in rows equidistant from the control. Isolates (approximately 100 from each soil) were obtained by use of nonselective plating and isolation procedures previously described (18, 20), and stock cultures maintained in soil extract yeast semisolid agar.

Synthesis of growth factors

In a recent study (19, 20) of the specific vitamin requirements of the predominant bacteria of a field soil the three vitamins found to be most essential were, in order of frequency, thiamine, biotin, and vitamin B_{12}. The ability of a total of 316 isolates to produce these substances was therefore studied, while the tests were extended to include recognition of the capacity of the organisms to produce riboflavin and the terregens factor.

For each isolate, three culture media of increasing complexity were used, the composition of which, per liter, is shown in table 1. The salts were first added to

TABLE 1

Composition of culture media for growth factor production

Ingredients (per liter)	Media			Ingredients (per liter	Media		
	1	2	3		1	2	3
K_2HPO_4.....*g.*	1.0	1.0	1.0	$CoCl_2 \cdot 6H_2O$..*mg.*	8	8	8
KNO_3.....*g.*	1.0	0.5	0.5	Sucrose.....*g.*	5.0	—	—
$MgSO_4 \cdot 7H_2O$....*g.*	0.2	0.2	0.2	Glucose.....*g.*	—	1.0	1.0
$CaCl_2$.....*g.*	0.1	0.1	0.1	"Casamino	—	4.0	4.0
NaCl.....*g.*	0.1	0.1	0.1	acids"*.....*g.*			
$FeCl_3 \cdot 6H_2O$....*mg.*	10	10	10	Yeast extract*.*g.*	—	—	1.0

* Difco.

distilled water and the solution adjusted to pH = 6.8, heated to boiling, cooled, and filtered, after which the other ingredients were added. The media were dispensed in 40-ml. quantities in 125-ml. Erlenmeyer flasks. Following inoculation from 5-day soil extract agar cultures the flasks were incubated in a reciprocal shaker for 5 days at 26°C.

Preparation of test samples of culture filtrates

From all cultures showing definite growth, which varied from slight to abundant, test samples of the metabolic fluids were prepared. The reaction was first adjusted to pH 6.4 and the flasks heated at 15 pounds pressure for 7 minutes to liberate bound vitamin. The cultures were then clarified by centrifugation and the clear liquids sterilized. In all 586 filtrates were so obtained.

Testing filtrates for growth factors

Tests for thiamine, biotin, and riboflavin were restricted to culture filtrates of medium 1 and medium 2 (table 1), both of which were vitamin-free; medium 3 included yeast extract, a source of the vitamins in question. Since vitamin B_{12} and the terregens factor are not, however, contained in yeast extract, filtrates from all three media were tested for the presence of B_{12} and TF.

The general procedure for recognition of the growth factors was as follows: add to 5.0 ml. of a suitable basal medium, adequate for growth of the test organism except for the factor in question, 0.5 ml. of the clarified metabolic liquid; inoculate with a loopful (1 mm.) of a suspension of the test organism; and incubate the tubes at 26°C. for 3 to 5 days. Negative and positive control media were inoculated in all cases. Definite growth of the test (indicator) organism in the tubes containing the filtrate was considered indicative of the presence of the growth factor in question.

The indicator organisms used were all isolated from soil in the course of previous investigations (3, 17, 20) and were chosen on the basis of their specific growth factor requirements. None showed response to combinations of other vitamins in the absence of the factor or factors found to be essential, nor did the addition of other vitamins improve the growth provided by the essential factor

TABLE 2

Indicator organisms and control media used in detection of growth factors in metabolic filtrates

Culture No.	Growth Factor Requirements*	Growth Factor Tested for	Negative Control Medium† in Addition to Salts and Glucose	Positive Control Medium (addenda to neg. cont.)
824 (*Arthrobacter* sp.)	thiamine (16)	thiamine	casamino acids	thiamine (50 μg./100 ml.)
54 (*Micrococcus* sp.)	biotin thiamine vitamin B_{12} (17)	biotin	casamino acids, thiamine, vitamin B_{12}	biotin (0.1 μg./100 ml.)
785 (*Flavobacterium* sp.)	riboflavin (16)	riboflavin	casamino acids	riboflavin (50 μg./100 ml.)
541 (*Arthrobacter* sp.)	vitamin B_{12} thiamine (16)	vitamin B_{12}	yeast extract (2nd control med.) yeast extract, TF	vitamin B_{12} (0.2 μg./100 ml.)
401 (*Arthrobacter* sp.)	terregens factor thiamine (3)	terregens factor	yeast extract (2nd control med.) yeast extract vitamin B_{12}	terregens factor (10 μg./100 ml.)

* Numbers in parentheses refer to REFERENCES.

† Basal medium to which metabolic filtrates added.

or factors under the experimental conditions. The cultures, as well as the control media used, are indicated in table 2.

RESULTS

The main findings are summarized in tables 3 and 4. As expected, the percentage of isolates able to grow increased with the complexity of the culture medium (table 3). Those unable to develop in medium 3, which contained yeast extract, comprised organisms that required vitamin B_{12} or the terregens factor. The lower relative incidence of such forms in the rhizosphere (5.7 per cent and 2.9 per cent compared with 8.4 per cent in the control soil) agrees with results of a previous study of these groups (14). With all soils the metabolic fluids of the richer media permitted recognition of the highest percentage of growth factors in all cases. It is therefore probable that somewhat larger numbers of isolates capable of producing thiamine, biotin, and riboflavin would have been recognized by the use of media containing vitamins other than those being tested but varying according to the special nutritional requirements of each isolate.

It was considered unlikely that the recognition of growth factors in the metabolic filtrates was obscured by the simultaneous production of an antibiotic, but to examine this possibility all filtrates of medium 3 which gave negative tests for both B_{12} and TF (177 in number) were tested. To 5.0-ml. portions of the respective positive control media 0.5 ml. filtrate was added. In no case did

TABLE 3

Growth factors produced in different media by isolates from control and rhizosphere soils

Culture Medium	Isolates Growing		Isolates in Culture Filtrate*									
			Thiamine		Biotin		Riboflavin		Vitamin B_{12}		Terregens factor	
	No.	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%	*No.*	%
Control Soil: 115.8 million/g.†; 107 isolates tested												
1	18	16.8	5	4.7	4	3.7	11	10.3	3	2.8	3	2.8
2	71	66.3	36	33.6	19	17.8	40	37.4	20	18.7	17	15.9
3	98	91.6	—	—	—	—	—	—	30	28.0	18	16.8
Rhizosphere (rye): 1156.6 million/g.†; 105 isolates tested												
1	13	12.4	3	2.9	1	1.0	7	6.7	2	1.9	1	1.0
2	76	72.4	33	31.4	16	15.2	36	34.3	18	17.1	7	6.7
3	99	94.3	—	—	—	—	—	—	32	30.5	17	16.2
Rhizosphere (barley): 2622.6 million/g.†; 104 isolates tested												
1	30	28.8	10	9.6	0	0.0	17	16.3	9	8.6	6	5.8
2	80	76.9	58	55.8	34	32.7	69	66.3	21	20.2	14	13.5
3	101	97.1	—	—	—	—	—	—	34	32.7	24	23.1

* Number of isolates and per cent of total isolates.

† Total count.

TABLE 4

Growth factors produced in one or more culture media by isolates from control and rhizosphere soils

Soil	Growth Factors Produced					
	Thiamine	Biotin	Riboflavin	Vitamin B_{12}	Terregens factor	One or more factors
Control (107 isolates):						
alone	1	0	2	8	5	
with others	37	21	40	24	19	
total	*38*	*21*	*42*	*32*	*24*	*59*
% of isolates	*35.5*	*19.6*	*39.2*	*29.9*	*22.4*	*50.5*
Rhizosphere-rye (105 isolates):						
alone	2	0	3	12	7	
with others	32	16	34	23	13	
total	*34*	*16*	*37*	*35*	*20*	*62*
% of isolates	*32.4*	*15.2*	*35.2*	*33.3*	*19.0*	*59.0*
Rhizosphere-barley (104 isolates):						
alone	1	0	5	11	2	
with others	58	34	65	25	24	
total	*59*	*34*	*70*	*36*	*26*	*85*
% of isolates	*56.7*	*32.7*	*67.3*	*34.6*	*25.0*	*81.7*

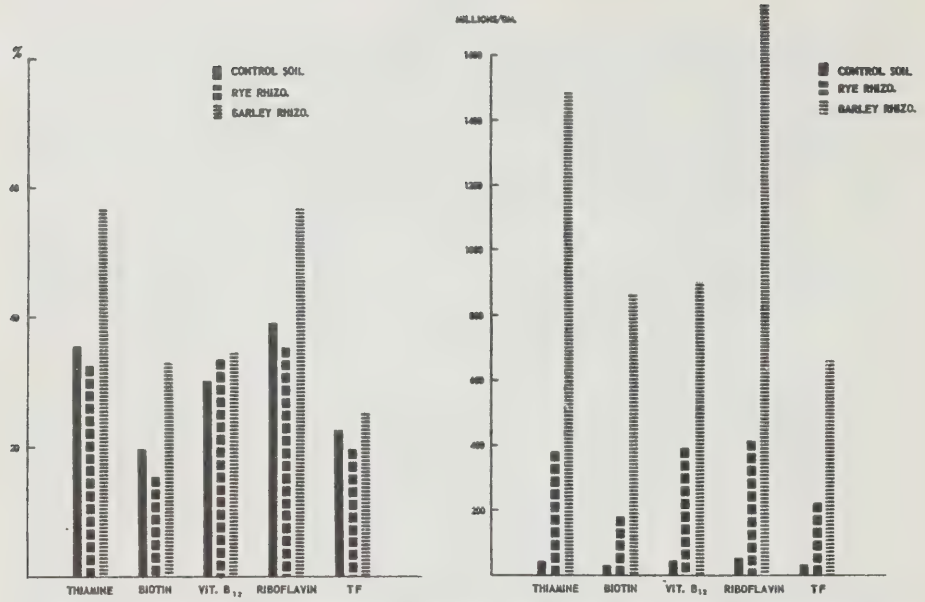

FIG. 1. PERCENTAGE INCIDENCE OF BACTERIA (*left*) AND ESTIMATED NUMBERS OF BACTERIA PER GRAM (*right*) CAPABLE OF SYNTHESIZING VARIOUS GROWTH FACTORS (CONTROL AND RHIZOSPHERE SOILS).

the presence of the metabolic fluid prevent development of the appropriate test organism although in a very few instances growth of culture 541, responding to B_{12}, was somewhat reduced.

The effect of plant growth on the percentage incidence of organisms producing the five growth factors and on their numbers per gram of soil, estimated from the total bacteria counts, is shown in figure 1. The proportion of bacteria found to produce B_{12} and TF showed little variation as between control and rhizosphere soils. With respect to thiamine, biotin, and riboflavin no "rhizosphere effect" was noted with rye; in the barley rhizosphere, however, the percentage of organisms found capable of synthesizing these vitamins was noticeably higher than in the control soil. In view of the pronounced increases in total counts, the numbers of growth factor synthesizing bacteria were much higher in the rhizospheres, and for thiamine, biotin, and riboflavin, particularly so in the rhizosphere of barley where the effect of the proportionate increases was emphasized (fig. 1, *right*). The significance that can be attributed to any *specific* crop effect, however, must await results of further studies.

DISCUSSION

The data presented indicate the potentialities of the indigenous soil bacteria for vitamin synthesis and do not establish that the organisms necessarily produce the vitamins in soil or produce them to the same degree. The findings do indicate, however, that they are able to produce them under appropriate

conditions and there is no cause to doubt that certain circumstances may provide suitable conditions in the soil. From the nature of the anabolic and catabolic reactions proceeding simultaneously it is obviously impossible to evaluate them separately in a natural soil. Though the vitamin levels may be estimated by appropriate assay methods, the amounts found represent only balances prevailing at any given time between closely interwoven synthetic and destructive agencies. The situation is further complicated by the utilization of one or more preformed vitamins required by organisms capable of synthesizing others.

The findings show that in the rhizosphere of plants the numbers of organisms with capabilities for vitamin synthesis may attain very high levels. In view of this it is difficult to avoid the conclusion that this potentiality may be of considerable significance, particularly in assessing the interrelated roles played by the growing plant, the normal soil microflora, and soil-borne plant pathogenic microorganisms.

When the findings are considered along with those relating to the occurrence in soil of high numbers of organisms for which growth-promoting substances are essential or stimulatory (14, 16), there is reason to believe that growth factors warrant far more consideration than has been accorded them in attempts to solve problems concerned with the mechanisms of plant root infection and disease control, and with an emphasis at least equal to that given to antagonistic phenomena.

In a valuable historical survey of root-disease investigation, Garrett (7) emphasized the failure which has attended attempts at biological control by procedures involving inoculation of the soil with antibiotic-producing microorganisms, something which, as he points out, might have been forecast from ecological considerations. It is now generally admitted that any effective biological control is more likely to be achieved by making environments more suited to antagonistic types rather than by inoculation, in itself. However, until the mechanisms of infection and control have been elucidated, any effective measures must be developed by empirical methods with their attendant limitations (7). It is reasonable to assume that such mechanisms must be studied with an awareness that growth-promoting as well as antibiotic effects, should be taken into consideration.

There is some weight of evidence, discussed by Garrett (8), which favors the hypothesis that germination of fungal spores may depend upon activating substances (2) and that in some cases the germinating activator may itself be a vitamin (10). The extension of this concept to problems of root rot control is suggested by the work of Mitchell, Hooton, and Clark (21) who made the important observation that additions of organic matter, which acted favorably in the control of root rot of cotton, stimulated the germination of sclerotia of *Phymatotrichum omnivorum*. This germination, which was followed by disappearance of sclerotia of the pathogen, seems to be an important factor in the disease-controlling effect, usually ascribed directly to microbial antagonism, and strongly suggests an activating, or growth-promoting action. That an analogous situation may prevail in the case of root rot of wheat caused by *Helminthosporium*

sativum is suggested by the findings of Chinn *et al.* (4, 5) who showed that certain amendments may, in the absence of the host plant, induce germination of dormant spores, followed by lysis of the germ tubes. Mechanisms involving stimulative effects do not appear to have received the attention they deserve.

The occurrence in the soil, and particularly in the rhizosphere, of large numbers of microorganisms which require growth factors and, apparently in even greater abundance, of those with capabilities for growth factor synthesis, points to the need for more precise knowledge of spore germination activators, growth-promoting exudates of roots, and factors required or produced by microorganisms (including pathogens) as related to their differential effects on saprophytic and parasitic forms in the soil and the plant itself. Even our assessment of antagonisms in soil may require revision in view of the capacity of certain antibiotics to be stimulatory at low concentrations (11, 12) for, in soil, concentrations may be very low and thus the laboratory antagonist may not only not be antagonistic in soil but exert a stimulatory action.

SUMMARY

A study was made of the ability of 316 cultures, comprising approximately equal numbers isolated, respectively, from a control field soil and the rhizosphere soils of rye and barley by nonselective procedures, to synthesize in various media five growth factors, namely thiamine, biotin, vitamin B_{12}, riboflavin, and the terregens factor.

With all three soils, riboflavin was produced by the highest percentage of isolates under the experimental conditions, followed by thiamine and vitamin B_{12}, somewhat lower proportions being found capable of synthesis of biotin and the terregens factor. In the majority of cases more than one factor was detected when any was produced. One-half the cultures from the control soil formed one or more growth factors and rather higher percentages of the rhizosphere isolates.

No rhizosphere effect was noted with either crop in respect to the percentage of isolates producing B_{12} or TF; with barley, though not with rye, the relative incidence of organisms forming riboflavin, thiamine, and biotin was greater than in the control soil. However, in view of the pronounced increase in total numbers of bacteria adjacent to the roots, the absolute numbers of organisms capable of producing growth factors were much higher in the rhizospheres.

The high incidence which bacteria capable of vitamin synthesis may attain in the rhizosphere suggests that this potentiality may be of significance in the interrelationships between the normal soil microflora, soil-borne plant pathogenic organisms, and the plant, and that the elucidation of mechanisms of infection and control will depend upon adequate consideration of growth-promoting as well as of antagonistic effects.

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